HERBAL PRODUCTS AND ESSENTIAL OILS WITH HIGH ACTIVITY AGAINST STATIONARY PHASE BARTONELLA HENSELAE

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ABSTRACT

Bartonella henselae is a Gram-negative bacterium which is the causative agent of cat scratch disease. Humans infection with *B. henselae* can result in acute or chronic systemic infections. The current antibiotic therapy to treat *Bartonella* infections is not very effective, presumably due to the bacterial persistence. This phenomenon gives rise to the importance of identifying more active drugs targeting bacterial persister cells to develop more effective therapies.

Herbal products and essential oils are plant extracts containing organic chemical compounds. Many botanical extracts and essential oil components have been documented to have antimicrobial activities. Nowadays it is important to study botanical products with potential antimicrobial activity, especially with the great concern about increasing antibiotic resistance.

In this study, we performed a high-throughput screening of two collections of herbal products and essential oils for active hits against stationary phase *B. henselae in vitro*. The primary screen was conducted using a SYBR Green I/propidium iodide (PI) viability

assay, followed by colony forming unit (CFU) assay throughout a seven-day drug exposure to confirm top hits. We successfully identified 3 herbal product extracts that had high activity against stationary phase *B. henselae* at 0.25% (*v/v*), derived from *Cryptolepis sanguinolenta*, *Juglans nigra*, and *Polygonum cuspidatum*. In addition, we identified 32 essential oils that had high activity against stationary phase *B. henselae*, including four essential oils extracted from *Citrus* plants, three from *Origanum*, three from *Cinnamomum*, two from *Pelargonium,* and two from *Melaleuca*, as well as frankincense, ylang-ylang, fir needle, mountain savory (winter), citronella, spearmint, elemi, vetiver, clove bud, allspice, and cedarwood essential oils. The time-kill assay showed 13 active hits could eradicate all stationary phase *B. henselae* in seven days at 0.032% (*v/v*). Two active ingredients, carvacrol and cinnamaldehyde, of oregano and cinnamon bark essential oils, respectively, were shown to be very active such that they were able to eradicate all the *B. henselae* cells even at ≤ 0.01% (*v/v*). The minimum inhibitory concentration (MIC) determination of these active hits indicated they also had

good activity against log phase growing *B. henselae*. These findings may have implications for developing more effective treatments for persistent *Bartonella* infections.

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INTRODUCTION

Bartonella henselae and its clinical significance

Bartonella species are fastidious, Gram-negative, facultative intracellular pathogens [1–3] with a unique intraerythrocytic lifestyle. These bacteria can be transmitted to humans or other mammalian hosts by several obligately bloodsucking arthropod vectors including fleas, sheep keds, lice, sand flies, ticks, and potentially mites and spiders [4]. So far, at least 40 species or subspecies of *Bartonella* have been discovered [5]. *Bartonella* bacteria can infect healthy people while being considered especially important as opportunistic pathogens [6]. At least 13 *Bartonella* species are known to be able to infect humans, causing either acute or chronic infections which could lead to cat scratch disease, endocarditis, bacillary angiomatosis, trench fever, Carrion's disease [3], bacteremia, central nervous system pathologies and so on [7]. This pathogenicity is partly due to their unique infection cycle including the lymphatic stage [8] and intraerythrocytic stage [4,9]. Three species of *Bartonella* including *B. henselae, B.*

quintana, and *B. bacilliformis,* are responsible for the great majority of infections in humans [10]. Among them, *B. henselae* is the most common zoonotic *Bartonella* species, with the infection distributed worldwide [11]. *B. henselae* is the causative pathogen of cat scratch disease, with symptoms of local skin lesions, malaise, decreased appetite, aches, headache, chills, arthritis, lymphadenopathy, fever with bacteremia, bacillary peliosis, and bacillary angiomatosis that could persist for several months [12-14]. In certain situations, cat scratch disease may lead to the development of serious neurologic or cardiac sequelae such as meningoencephalitis, seizures or endocarditis, which can have a particularly high mortality [15]. *B. henselae* could also serve as a co-infection pathogen with Lyme disease agent *B. burgdorferi* transmitted by ticks carrying multiple pathogens, leading to more severe and protracted clinical manifestations [16].

It is laborious using classical bacteriological methods to isolate and culture *Bartonella* spp. in liquid media especially from clinical samples, which requires specific conditions and prolonged incubation periods [17,18]. Therefore, serology and real-time

PCR are often used instead of culture to confirm the diagnosis for rapid *Bartonella* detection clinically [17,18]. The first-line antibiotics for treating bartonellosis include doxycycline, erythromycin, gentamicin, rifampicin, azithromycin, and ciprofloxacin, as well as some drug combinations like doxycycline plus gentamicin, doxycycline plus rifampin [19,20]. However, a systematic review has revealed that the current clinical treatment of *Bartonella* infections relies mostly on personal experience and expert opinion. The treatment lacks evidence of randomized trials and the recommended antibiotic treatment for cat scratch disease, infectious endocarditis, and bacillary angiomatosis showed no improvement in cure rate or cure time [21]. In particular, there is no single treatment effective for systemic *B. henselae* infections, and antibiotic therapy exhibited poor activity against typical uncomplicated cat scratch disease [19,20]. Therefore, bartonellosis treatment remains a significant problem and, without treatment, it could cause high mortality in some patients. This difficult situation could partly be attributed to bacterial persistence and bacterial biofilms which are resistant or tolerant to antibiotic treatments and can evade host immune defense [22].

Bacterial persistence of *Bartonella henselae*

According to the Yin-Yang model theory [23], the bacterial population is highly heterogeneous which consists of growing (Yang) and non-growing persister (Yin) cells that are in varying growth and metabolic states in continuum. Persister cells may escape the effects of antibiotics due to epigenetic changes [24]. Therefore, Yang growing cells could cause active diseases at the host level, while Yin persister cells could remain dormant in the host during antibiotics exposure without being eradicated and revert to growing forms under appropriate conditions, which could lead to relapse or prolonged infections with symptoms [23]. The number of persister cells in a growing population of bacteria rises in the mid-log growth phase, with a maximum of 1% of the bacterial population being persisters when cultures reach the stationary phase [25]. As described by the Yin-Yang model, persisters can adopt varying sizes and shapes from regular morphology to altered morphologies as found in biofilms and L-form bacteria, and there would be a substantial number of persisters formed within a biofilm structure [26-28], which are not killed easily by current antibiotics.

B. henselae is capable of growing as aggregates and forms a biofilm structure on infected native and prosthetic heart valves. It is frequently found in the heart valve vegetations of patients with blood-culture-negative endocarditis [3,29]. The discrepancy between *in vitro* antibiotic susceptibility data indicated by low MICs and the failure of antibiotic therapy *in vivo* demonstrates the clinical significance of *B. henselae* persistence [30], because the *in vitro* tests were mainly based on growing bacteria. A previous study has summarized that *B. henselae* has a substantial capacity to tolerate antimicrobial agents due to bacterial persistence and biofilm formation which pose significant challenges for treatment [31]. Therefore, identifying drugs that target *Bartonella* persister cells in the stationary phase or biofilms could provide a promising strategy for developing a more effective treatment for *Bartonella* infections.

Herbal products and essential oils as potent antimicrobials

The use of botanical medicines was documented in the ancient cultures of Mesopotamia, China and India, and their safety and efficacy were demonstrated by

various traditional medicine systems such as Ayurveda and traditional Chinese medicine [32-34]. The adverse effects of botanical products were determined to be rare according to recent reports [35-37]. Many botanical extracts have been reported to have antimicrobial activities, for example, *Laserpitium zernyi* herb extracts were active against many kinds of bacteria including *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Enterococcus faecalis* and *Bacillus subtilis*, different extracts of *Ononis arvensis* showed antimicrobial activity against *Escherichia coli*, *P. aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Candida albicans*, and some Mediterranean herb extracts could inhibit microbial growth of representative oral microorganisms and biofilm formation of *Streptococcus mutans* [38-40]. Nowadays it's worth studying herbs with potential antimicrobial activity for improved treatment development, especially with the great concern about increasing antibiotic resistance.

The essential oil, also known as volatile oil or ethereal oil, is a concentrated hydrophobic liquid containing volatile chemical compounds extracted from plants. It has many uses in aromatherapy [41], food processing [42], and also potentially in medical

therapy [43]. Previous *in vitro* studies have found certain essential oils had antibacterial activity against multidrug-resistant Gram-negative clinical isolates [44]. In fact, many essential oil compositions including carvacrol, thymol, cinnamic acid, trans-cinnamaldehyde, eugenol, α-pinene, and γ-terpinene were documented decades ago to have antimicrobial activities. Some essential oil components were shown to have a synergistic effect in combination with antibiotics [45]. Therefore, essential oils could serve as a reservoir of potentially powerful antimicrobials.

An optimized SYBR Green I/PI assay for rapid viability assessment for *B. henselae*

One conventional antibiotic susceptibility test is the Kirby–Bauer disk diffusion assay for growing bacteria, and the colony forming unit (CFU) assay is the most commonly used method to assess the killing activity of antibiotics against stationary phase bacteria. The residual bacterial viability after drug exposure is assessed by counting viable cells grown on agar plates in CFU assay. However, major disadvantages of CFU

assay include the laborious procedure to wash cells and make serial dilutions, the lengthy time for bacteria to grow on agar plates which sometimes requires several days or weeks, and the possibility of missing the subpopulation of viable but non-culturable bacteria that do not form CFUs. In order to expedite the process of antimicrobial activity testing, we first developed a novel approach using a SYBR Green I/Propidium iodide (PI) staining viability assay for high-throughput drug screens of *Borrelia burgdorferi* [46]. SYBR Green I is a high affinity dye that binds double-stranded DNA (dsDNA) and is commonly used to stain nucleic acids in polymerase chain reaction (PCR) and flow cytometric analysis [47-49], which stains all live cells green in the SYBR Green I/PI assay. PI is an impermeable dye that only stains dead or damaged cells with compromised cell membrane red [50]. Therefore, we could measure the live/dead ratio of a bacterial sample after drug exposure by green/red fluorescence ratio calculated through fluorescence microscopy or fluorescence microplate reader, which provides a rapid method for screening a large drug library without depending on CFU counts.

Our previous studies have used this SYBR Green I/PI assay for the identification of many herbal products and essential oils with high activity against stationary phase *Borrelia burgdorferi* [51-53] as a surrogate model of persister bacteria [54]. We have also successfully adapted this SYBR Green I/PI assay for the rapid viability assessment of *B. henselae* and have successfully used this assay for high-throughput drug screens against non-growing stationary phase *B. henselae* using the FDA drug library. We used this library to measure the linear relationship between the live/dead bacterial cell ratio and the green/red fluorescence ratio, and generated a regression equation and regression curve [55]. Here, we adapted the same SYBR Green I/PI methodology to perform an efficient screen using our herbal product and essential oil collections against stationary phase *B. henselae* and successfully identified a significant number of herbal products and essential oils that had good activity against non-growing *B. henselae* cells. We have also identified two essential oil ingredients to be highly active against stationary phase *B. henselae.* The implication of the identified active hits for improved treatment of persistent *Bartonella* infections is discussed.

MATERIALS AND METHODS

Bacterial strain, culture media and growth conditions

The *Bartonella henselae* JK53 strain was obtained from BEI Resources (ATCC), NIAID, NIH. Based on the culture medium developed in a previous study [56], *B. henselae* JK53 was cultured in Schneider's Drosophila medium (Life Technologies Limited, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Co., St. Louis, MO, USA) and 5% sucrose (Fisher Scientific, New Jersey, USA). Cultures were incubated in sterile 15 mL or 50 mL polypropylene conical tubes (Corning, New York, USA) in microaerophilic incubator without shaking at 37℃, 5% CO_2. As previously measured [55], *B. henselae* JK53 rapidly went into the logarithmic growth phase in one day and reached a growth peak after two days under such culture conditions. The one-day-old and five-day-old culture were considered as log phase and stationary phase, respectively. The Columbia anaerobic sheep blood agar (HemoStat

Laboratories, Dixon, CA, USA) was used to perform the colony forming unit (CFU) enumeration for the drug exposure assay, which was also cultured at 37 °C, 5% CO_2.

Preparation of antibiotics, herbal products, essential oils, and their active ingredients

Antibiotics including azithromycin (AZI), daptomycin (DAP), doxycycline (DOX), gentamicin (GEN), methylene blue, miconazole, moxifloxacin (MXF), nitrofurantoin (NIT), and rifampin (RIF) were purchased from Sigma-Aldrich (USA) and dissolved in appropriate solvents [57] to form 10 mg/mL or 100 mM stock solutions. All the antibiotic stocks were filter-sterilized by 0.2 μm filters except the dimethyl sulfoxide (DMSO) stocks and then diluted and stored at −20 °C.

A panel of 18 herbal products was purchased from KW Botanicals Inc. (San Anselmo, CA, USA) and Heron Botanicals (Kingston, WA, USA). These botanical products were chosen based on significant antimicrobial activity against other bacterial pathogens shown by previous studies [58-62], anecdotal clinical usage reported by

patients or herbalists, favorable safety profiles and good absorption systemically. Botanicals were identified via macroscopic and organoleptic methods, and voucher specimens were on file with the respective production facilities. Additional details on sourcing, testing and validation of botanical and natural medicines used are summarized in Table S1. Most botanical extracts were provided as water extract or as 30%, 60%, and 90% alcohol extracts, and the alcohol used was also tested separately as a control in the same dilutions of 30%, 60%, and 90%. Herbal products were dissolved in DMSO at 5% (*v/v*), followed by dilution at 1:5 into five-day-old stationary phase *B. henselae* JK53 cultures to achieve 1% final concentration. To make further dilutions for evaluating anti-*Bartonella* activity, the 1% herb products were further diluted with the same stationary phase cultures to achieve desired concentrations.

A panel of 149 essential oils was purchased from Plant Therapy (ID, USA), Natural Acres (MO, USA), or Plant Guru (NJ, USA). Detailed information, as well as some GC-MS reports of these essential oils, are available at the vendors' websites. The main chemical compositions of active essential oils are summarized in Table S2 based on

vendors' GC-MS reports or previous studies. Carvacrol and cinnamaldehyde were purchased from Sigma-Aldrich (USA). DMSO-soluble essential oils and carvacrol and cinnamaldehyde were dissolved in DMSO at 5% (*v/v*), followed by dilution at 1:10 into five-day-old stationary phase cultures to achieve 0.5% final concentration. To make further dilutions for evaluating anti-*Bartonella* activity, the 0.5% essential oil treatments were further diluted in the same stationary phase cultures to achieve desired concentrations. DMSO-insoluble essential oils were added directly to five-day-old stationary phase *B. henselae* cultures to form emulsion by adequate vortexing, followed by immediate transfer of the emulsion into the same stationary phase cultures to make serial dilutions to achieve desired final concentrations.

Microscopy techniques

Drug-treated or control *B. henselae* cell suspensions were stained with the SYBR Green I (100 × stock, Invitrogen) and propidium iodide (PI, 600 μM, Sigma) mixture dye dissolved in distilled water and then examined with BZ-X710 All-in-One fluorescence

microscope (KEYENCE, Inc., Osaka, Japan). The SYBR Green I/PI dye was added to the drug-treated sample at 1:10 dilution and mixed thoroughly, followed by incubating in the dark at room temperature for 15 minutes. Then the residual bacterial viability could be assessed by calculating the ratio of green/red fluorescence, respectively, as described previously [46]. The residual bacterial viability was confirmed by analyzing three representative images of the same bacterial cell suspension using the fluorescence microscopy. BZ-X Analyzer (provided by Keyence) and Image Pro-Plus software were used to quantitatively determine the fluorescence intensity.

Screening of herbal products and essential oil collection against stationary phase *B. henselae* by SYBR Green I/PI viability assay

For the primary high-throughput herbal products screen, each product was assayed in two concentrations, 1% (*v/v*) and 0.5% (*v/v*). A five-day-old stationary phase *B. henselae* JK53 culture was used for the primary screen. Firstly, 40 µL 5% herbal product DMSO stocks were added to 96-well plate containing 160 µL *B. henselae*

culture, respectively, to obtain the desired concentration of 1%. Then the 0.5% concentration was obtained by mixing 100 μL 1% treatment with 100 μL *B. henselae* culture. Antibiotics including AZI, DAP, DOX, GEN, methylene blue, miconazole, and RIF were used as control drugs at their Cmax. Control solvents including DMSO, 30%, 60%, and 90% alcohol were also included. Plates were sealed and placed in 37°C incubator without shaking over of a period of three days. SYBR Green I/ PI viability assay was then used to assess the live/dead cell ratios after drug exposure as described [55]. Briefly, the SYBR Green I/PI dye was added to the sample followed by incubation in the dark for 15 minutes. The concentration of the mixed dye was consistent with that described above. With excitation wavelength of 485 nm and 538 nm and 650 nm for green and red emission, respectively, the green and red fluorescence intensity was determined for each sample using a microplate reader (HTS 7000 plus Bioassay Reader, PerkinElmer Inc., USA). Then the residual bacterial viability was calculated according to the regression equation of the relationship between residual

viability percentage and green/red fluorescence ratio obtained by least-square fitting analysis as previously described [55]. All tests were run in triplicate.

For the primary high-throughput essential oil screen, each essential oil was assayed in two concentrations, 0.5% (*v/v*) and 0.25% (*v/v*). Firstly, 20 µL 5% essential oil DMSO stocks or emulsion were added to the 96-well plate containing 180 µL of the five-day-old stationary phase *B. henselae* JK53 culture to obtain the desired concentration of 0.5%. Then, the 0.25% treatment concentration was obtained by mixing 100 µL 0.5% treatment with 100 µL five-day-old *B. henselae* JK53 culture. Antibiotics including AZI, DAP, DOX, GEN, methylene blue, miconazole, MXF, NIT, and RIF were used as control drugs at 20 µM. Plates were sealed and placed in a 37 °C incubator without shaking over a period of three days. SYBR Green I/ PI viability assay was used to assess the live and dead cells after drug exposure as described above. All tests were run in triplicate.

Drug exposure assay of active hits by CFU counting on agar plates

Based on primary screening results, active hits were further confirmed by the drug exposure CFU assay. The five-day-old stationary phase *B. henselae* culture was used for drug exposure experiments, which was treated with 0.25% (*v/v*) active herbal products or 0.032% (*v/v*) active essential oils respectively. Then two highly potent active ingredients, carvacrol, and cinnamaldehyde, of active essential oils oregano and cinnamon bark, respectively, were also tested by drug exposure assay at a very low concentration of 0.01% (*v/v*) and 0.005% (*v/v*). Control antibiotics including AZI, DAP, DOX, GEN, methylene blue, miconazole, and RIF were used at their Cmax. Solvents including DMSO, 30%, 60%, and 90% alcohol were also included as controls for herbal products assay. The drug exposure assay was carried out in 15 mL polypropylene conical tubes over the course of seven days at 37 °C, 5% CO_2 without shaking. At each time point we measured, a portion of bacteria cells was collected by centrifugation and rinsed twice with fresh Schneider's medium followed by resuspension in fresh Schneider's medium. Then the cell suspension was serially diluted and each dilution

was plated on Columbia blood agar plates for viable bacterial CFU counts. The plates were incubated at 37 °C, 5% CO_2 until visible colonies appeared and the CFU/mL was calculated accordingly. All tests were run in triplicate.

Minimum inhibitory concentration (MIC) determination of active hits

The standard microdilution method was used to measure the minimum inhibitory concentration (MIC) of each active herbal product or essential oil needed to inhibit the visible growth of *B. henselae* after a five-day incubation period as described [55]. The diluted one-day-old logarithmic phase *B. henselae* culture was used for MIC determination. 1×10^6 bacterial cells were inoculated into the well of the 96-well plate containing 160 µL fresh modified Schneider's medium per well. Then 40 µL 5% herbal product or essential oil stocks were added into each well respectively to achieve 1% final concentration. Other lower concentrations were obtained by mixing 1% treatment with diluted one-day-old log phase *B. henselae* culture. Plates were sealed and incubated at 37 °C without shaking for five days. Then the bacterial cell proliferation was

assessed using the SYBR Green I/PI assay as described above and the bacterial counting chamber after the incubation. The MIC is the lowest concentration of the drug that prevented the visible growth of *B. henselae*. All tests were run in triplicate.

Statistical Analysis

The statistical analysis was performed using two-tailed Student's *t*-test and two-way ANOVA where appropriate. Mean differences were considered statistically significant if p was <0.05. All experiments were performed in triplicate. Analyses were performed using Image Pro-Plus, GraphPad Prism, and Microsoft Office Excel.

RESULTS

I. Evaluation of herbal product collection for activity against *B. henselae*

Screening the herbal product collection to identify active hits against non-growing stationary phase *B. henselae*

Previously, we have developed an SYBR Green I/PI viability assay for the rapid viability assessment of *B. henselae* and have successfully used this assay for high-throughput drug screens against non-growing stationary phase *B. henselae* using the FDA-approved drug library [55]. Although the standard CFU assay can be used to evaluate the activity of different drugs against stationary phase *B. henselae*, it would be much more tedious. Thus, we screened the FDA drug library by using the SYBR Green I/PI assay which is a more rapid method with the potential to be used for more high-throughput drug screens [55]. Here we adapted this SYBR Green I/PI viability assay for herbal product screens against *B. henselae*. As described in the previous study [55], a

five-day-old stationary phase *B. henselae* culture was used to identify active herbal products against stationary phase *B. henselae*. All herbal products were applied at two concentrations, 1% (*v/v*) and 0.5% (*v/v*), respectively, in the primary screens, along with their solvents tested separately for comparison. Meanwhile, the currently known effective antibiotics used to treat bartonellosis such as AZI, DOX, GEN, and RIF were included as control drugs for comparison (Table 1). In addition, we included previously identified FDA-approved drugs that were effective against stationary phase *B. henselae* such as DAP, methylene blue, and miconazole [55] as controls (Table 1). All these antibiotics were used at their Cmax. In the primary screens, after the three-day drug exposure, 7 of the 18 herbal products in the collection were found to have good activity against stationary phase *B. henselae* both at the concentration of 1% (*v/v*) and 0.5% (*v/v*) as shown by plate reader results, and thus were selected as top hits. The top 7 active hits included *Juglans nigra* fruit extract, *Juglans nigra* 30%, 60%, and 90% alcohol extract, two different extracts from *Polygonum cuspidatum*, *Cryptolepis sanguinolenta* 30%, 60%, and 90% alcohol extract, *Scutellaria baicalensis*, and

Scutellaria barbata. These top hits were chosen based on their lower percentage of viable cells remaining after herbal product treatment than that for the current antibiotics used to treat *Bartonella* infections, including AZI, DOX, GEN, and RIF. According to our previous experience, some compounds in the herbal products can cause interference with the SYBR Green I/PI assay because of color and autofluorescence. Thus, we selected these 7 top hits for further validation by microscopic counting to confirm the SYBR Green I/PI plate reader results. The currently used antibiotics for bartonellosis treatment and the identified FDA-approved drugs effective against *B. henselae* were also included as controls for comparison. AZI and DOX as control drugs showed poor activity against stationary phase *B. henselae* (residual viability above 60%) (Table 1), as expected. Antibiotics reported to have a clinical improvement for *Bartonella* infection including GEN and RIF [30,63] showed relatively better activity (residual viability below 50%) against stationary phase *B. henselae* than AZI and DOX. FDA-approved drugs that we identified as effective against stationary phase *B. henselae* (DAP, methylene blue, and miconazole) had better activity (residual viability below 40%) than most of the

four antibiotics mentioned above. The difference of residual viabilities of stationary phase *B. henselae* after treatment by control solvents and without drug treatment were not statistically significant.

Table 1. Activity of top 7 herbal products active against stationary phase *B. henselae* [1]

Herbal Products (HP) and Control Drugs	Plant or Ingredients of Herbal Products	Residual Viability (%) after 1% HP or Antibiotic Treatment		Residual Viability (%) after 0.5% HP Treatment	
		Plate Reader[2]	Microscope[3]	Plate Reader[2]	Microscope[3]
Drug free control		70%	75%		
DMSO control		60%	70%		
30% alcohol		70%	78%		
60% alcohol		80%	82%		
90% alcohol		65%	63%		
AZI		70%	65%		
DOX		66%	60%		
GEN		44%	50%		
RIF		31%	45%		
DAP		6%	10%		
Methylene Blue		29%	35%		
Miconazole		40%	50%		
Hu tao ren	*Juglans nigra* fruit (husk/hull)	0%[4]	0%	9%	59%
Black walnut 60% AE[5]	*Juglans nigra*	0%[4]	0%	4%	32%
Black walnut 90% AE[5]	*Juglans nigra*	0%[4]	7%	6%	30%
Black walnut 30% AE[5]	*Juglans nigra*	2%	2%	6%	63%
Japanese knotweed	*Polygonum cuspidatum*	6%	0%	8%	21%
Hu zhang	*Polygonum cuspidatum*	8%	18%	13%	70%
Cryptolepis 30% AE[5]	*Cryptolepis sanguinolenta*	8%	10%	14%	42%
Huang qin	*Scutellaria baicalensis*	8%	12%	11%	25%

Ban zhi lian	*Scutellaria barbata*	8%	50%	2%	48%
Cryptolepis 60% AE[5]	*Cryptolepis sanguinolenta*	10%	24%	14%	34%
Cryptolepis 90% AE[5]	*Cryptolepis sanguinolenta*	11%	25%	14%	47%

[1] A five-day-old stationary phase *B. henselae* culture was treated with herbal products (1% or 0.5%) (*v/v*) or control drugs for three days. Drug concentrations used in this experiment were based on their Cmax and were as follows: 0.2 μg/mL AZI, 2.4 μg/mL DOX, 10 μg/mL GEN, 7.8 μg/mL RIF, 60 μg/mL DAP, 2.9 μg/mL methylene blue, and 6.3 μg/mL miconazole. [2] Residual viability was calculated according to the regression equation and the ratio of Green/Red fluorescence obtained by SYBR Green I/PI assay. [3] Residual viability was assayed by fluorescence microscope counting. [4] Values of SYBR Green I/PI calculated by the plate reader were lower than 100% dead cells. [5]Abbreviation: AE: alcohol extract.

Among the 7 top hits that had better activity (residual viability between 0% and 16%) against stationary phase *B. henselae* than most control antibiotics, the most active herbal products were extracts from *Juglans nigra* and *Polygonum cuspidatum*, including hu tao ren, black walnut alcohol extracts of different concentrations, Japanese knotweed, and hu zhang. However, the fluorescence microscope observation of hu tao ren, black walnut 30% AE, and hu zhang at 0.5% treatment exhibited significantly higher percentage of green (live) cells compared with the plate reader results (Figure 1)(Table 1), which were also higher than that of most control antibiotics, indicating the relatively

poor accuracy of the plate reader results and poor activity of these herbal products at these particular herbal concentrations. Therefore, they were excluded from active hits for subsequent MIC testing and drug exposure assay (see below). Alcohol extracts from *Cryptolepis sanguinolenta* of different concentrations also exhibited strong activity against stationary phase *B. henselae* as shown by red (dead) cells in fluorescence microscope observation (Figure 1), which is consistent with the plate reader results. Extracts from two plants of *Scutellaria* genus, including *Scutellaria baicalensis* (huang qin) and *Scutellaria barbata* (ban zhi lian), also showed remarkable effects with low percentages of residual viable bacterial cells remaining (Figure 1). Control drugs including AZI and DOX exhibited poor activity against stationary phase *B. henselae* as shown by many green (live) cells in fluorescence microscope observation, and other antibiotics including GEN, RIF, DAP, methylene blue, and miconazole showed better activity but not as good as most of the 7 top hits (Figure 1).

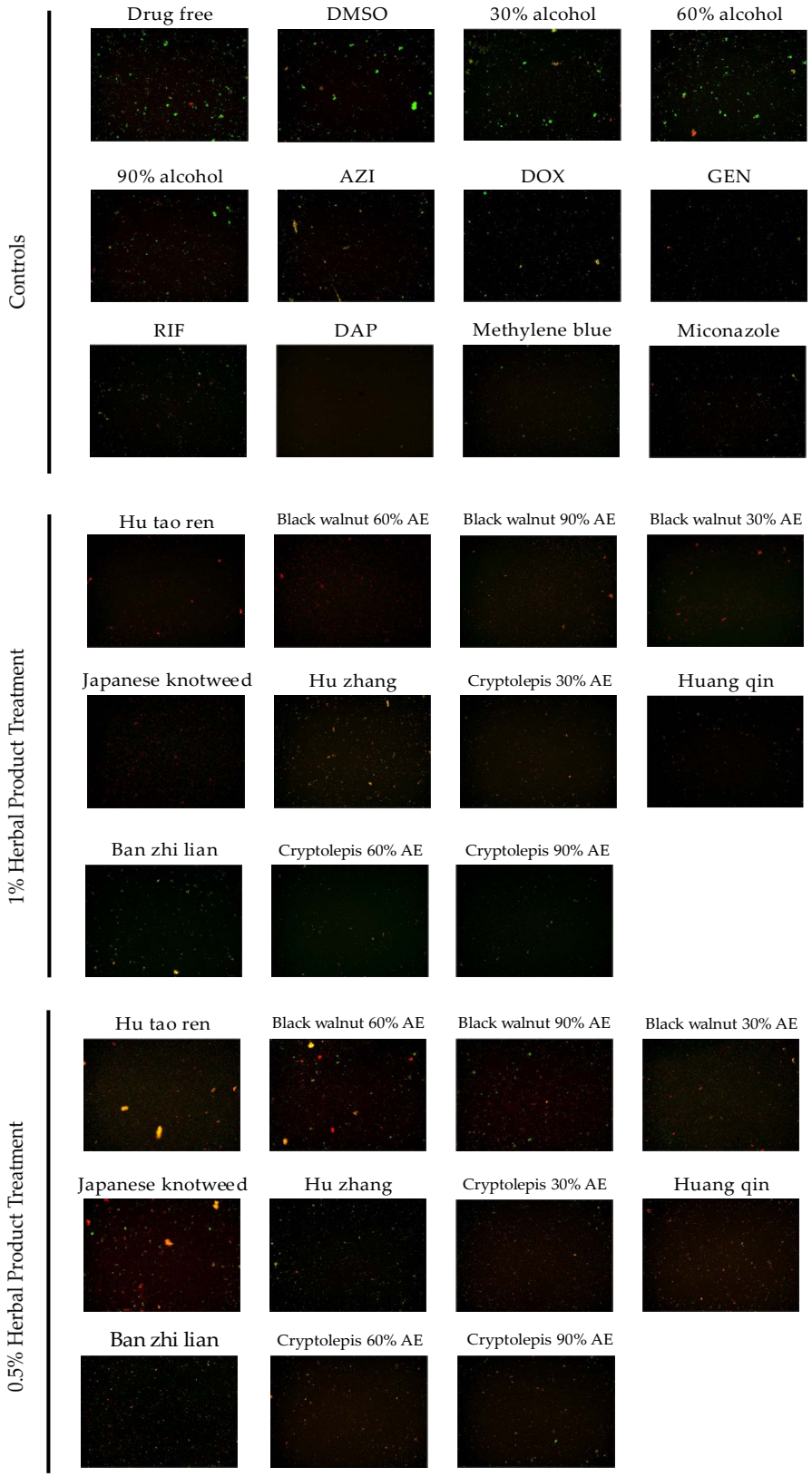

Figure 1. Effect of 7 top hits of herbal products against stationary phase *B. henselae* in comparison with control drugs. A five-day-old stationary phase *B. henselae* culture was treated with 1% (*v/v*) or 0.5%

(*v/v*) herbal products or control antibiotics for three days followed by SYBR Green I/PI viability assay and fluorescence microscopy (400 × magnification). Drug concentrations used in this experiment were based on their Cmax and were as follows: 0.2 μg/mL AZI, 2.4 μg/mL DOX, 10 μg/mL GEN, 7.8 μg/mL RIF, 60 μg/mL DAP, 2.9 μg/mL methylene blue, and 6.3 μg/mL miconazole. Green cells represent live cells and red cells represent dead cells.

MIC determination of active hits in the herbal product collection

The activity of antibiotics against non-growing bacteria is not always correlated with that against growing bacteria [55]. Thus, it was also necessary to determine the MICs of these active herbal products against log phase growing *B. henselae*. The MIC determination of herbal products for *B. henselae* was conducted by the standard microdilution method, as described in our previous study [55]. As shown in Table 2, black walnut 60% alcohol extract was the most active herbal product among the top 7 hits, capable of inhibiting visible *B. henselae* proliferation at the concentration of 0.125%-0.25% (*v/v*). Other herbal products including black walnut 90% alcohol extracts, Japanese knotweed, cryptolepis 30%, 60%, and 90% alcohol extracts, huang qin, and ban zhi lian had similar activity against growing *B. henselae* that they inhibited log

phase *B. henselae* proliferation at the concentration of 0.25%-0.5% (*v/v*). These results indicated that these top hits of herbal products were not only active against non-growing stationary phase *B. henselae*, but also effective against log phase growing *B. henselae.*

Table 2. Minimum inhibitory concentrations (MICs) of top active herbal products against *B. henselae*

Natural Products	Plant or Ingredients of Herbal Products	MIC (*v/v*)
Black walnut 60% AE	*Juglans nigra*	0.125%-0.25%
Black walnut 90% AE	*Juglans nigra*	0.25%-0.5%
Japanese knotweed	*Polygonum cuspidatum*	0.25%-0.5%
Cryptolepis 30% AE	*Cryptolepis sanguinolenta*	0.25%-0.5%
Cryptolepis 60% AE	*Cryptolepis sanguinolenta*	0.25%-0.5%
Cryptolepis 90% AE	*Cryptolepis sanguinolenta*	0.25%-0.5%
Huang qin	*Scutellaria baicalensis*	0.25%-0.5%
Ban zhi lian	*Scutellaria barbata*	0.25%-0.5%

Time-kill curves of active hits from the herbal product collection

To further demonstrate the efficacy of the active herbal products identified from the primary screens in eradicating non-growing *B. henselae* persistent cells, we performed

a time-kill drug exposure assay against a five-day-old *B. henselae* stationary phase culture at a low concentration of 0.25% (*v/v*), along with their corresponding solvent controls. Meanwhile, clinically used antibiotics to treat *Bartonella* infections including AZI, DOX, GEN, and RIF were used at their Cmax as controls. Compared to the drug free control, as shown in Figure 2 and Table 3, some clinically used antibiotics such as azithromycin and doxycycline showed poor activity in killing stationary phase *B. henselae* partly due to their low Cmax. Other antibiotics such as GEN and RIF exhibited better activity which could eradicate all *B. henselae* cells by day 7 and day 5. The difference of residual viabilities of stationary phase *B. henselae* after treatment by control solvents and without drug treatment were not statistically significant. All three cryptolepis alcohol extracts of different concentrations were able to eradicate all *B. henselae* cells in the seven-day drug exposure, where cryptolepis 60% alcohol extract was the most active herbal product that killed *B. henselae* with no detectable CFU after five-day exposure. Black walnut in 60% and 90% alcohol extracts both exhibited good activity that eradicated all *B. henselae* cells without viable cells being recovered after

the seven-day drug exposure. Japanese knotweed was also effective to kill all *B. henselae* cells by day 7. However, ban zhi lian and huang qin herbal products showed poor activity at the concentration of 0.25% (*v/v*) during this seven-day drug exposure, with considerable numbers of residual viable cells remaining after treatment.

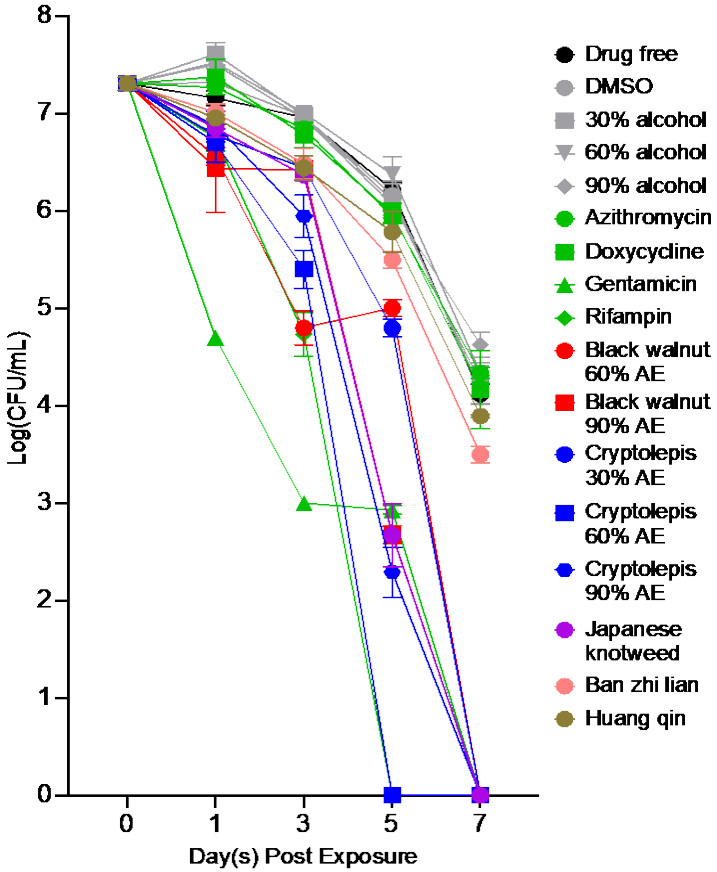

Figure 2. Time-kill curves of active herbal products treatment against stationary phase *B. henselae* in comparison with control drugs. The herbal products or control antibiotics were added to the five-day old stationary phase culture respectively at time point 0, and at different times of drug exposure (day 1, day 3, day 5, and day 7), portions of bacteria were removed and washed and plated on Columbia blood agar

plates for CFU counts. The herbal product concentration used in this experiment was 0.25% (*v/v*). Drug concentrations used in this experiment were based on their Cmax and were as follows: 0.2 µg/mL AZI, 2.4 µg/mL DOX, 10 µg/mL GEN, and 7.8 µg/mL RIF.

Table 3. Drug exposure assay of top active herbal products against *B. henselae* stationary phase culture [1]

Herbal Products and Control Drugs[2]	CFU/mL after Drug Exposure			
	1 Day	3 Day	5 Day	7 Day
Drug free control	$1.45 \pm 0.26 \times 10^7$	$9.17 \pm 1.44 \times 10^6$	$1.67 \pm 0.29 \times 10^6$	$1.33 \pm 0.29 \times 10^4$
DMSO	$2.07 \pm 0.12 \times 10^7$	$9.33 \pm 0.58 \times 10^6$	$1.50 \pm 0.50 \times 10^6$	$1.50 \pm 0.50 \times 10^4$
30% alcohol	$4{,}17 \pm 1.04 \times 10^7$	$9.50 \pm 1.32 \times 10^6$	$1.33 \pm 0.58 \times 10^6$	$1.83 \pm 0.76 \times 10^4$
60% alcohol	$3.33 \pm 0.29 \times 10^7$	$1.02 \pm 0.13 \times 10^7$	$2.50 \pm 1.00 \times 10^6$	$2.17 \pm 0.29 \times 10^4$
90% alcohol	$3.17 \pm 0.29 \times 10^7$	$9.00 \pm 1.50 \times 10^6$	$1.17 \pm 0.29 \times 10^6$	$4.33 \pm 1.15 \times 10^4$
AZI	$1.87 \pm 0.32 \times 10^7$	$7.00 \pm 1.00 \times 10^6$	$8.83 \pm 1.44 \times 10^5$	$2.17 \pm 0.29 \times 10^4$
DOX	$2.50 \pm 1.00 \times 10^7$	$6.17 \pm 1.76 \times 10^6$	$9.67 \pm 2.47 \times 10^5$	$1.83 \pm 1.15 \times 10^4$
GEN	$5.00 \pm 0.00 \times 10^4$	$1.00 \pm 0.00 \times 10^3$	$8.50 \pm 0.87 \times 10^2$	0
RIF	$5.83 \pm 1.76 \times 10^6$	$5.83 \pm 2.57 \times 10^4$	0	0
Black walnut 60% AE	$3.67 \pm 0.76 \times 10^6$	$6.67 \pm 2.89 \times 10^4$	$1.02 \pm 0.21 \times 10^5$	0
Black walnut 90% AE	$3.83 \pm 3.69 \times 10^6$	$2.67 \pm 0.58 \times 10^6$	$4.83 \pm 1.04 \times 10^2$	0
Cryptolepis 30% AE	$6.00 \pm 0.87 \times 10^6$	$2.83 \pm 0.76 \times 10^6$	$6.33 \pm 1.26 \times 10^4$	0
Cryptolepis 60% AE	$5.33 \pm 2.25 \times 10^4$	$2.67 \pm 1.04 \times 10^5$	0	0
Cryptolepis 90% AE	$7.83 \pm 2.75 \times 10^6$	$9.50 \pm 3.97 \times 10^5$	$2.17 \pm 1.04 \times 10^2$	0
Japanese knotweed	$7.17 \pm 1.61 \times 10^6$	$2.33 \pm 0.29 \times 10^6$	$5.50 \pm 3.12 \times 10^2$	0
Ban zhi lian	$1.03 \pm 0.20 \times 10^7$	$3.17 \pm 1.04 \times 10^6$	$3.17 \pm 0.58 \times 10^5$	$3.17 \pm 0.58 \times 10^3$
Huang qin	$9.00 \pm 0.50 \times 10^6$	$2.83 \pm 0.76 \times 10^6$	$6.50 \pm 2.78 \times 10^5$	$7.83 \pm 0.29 \times 10^3$

[1] A five-day-old stationary phase *B. henselae* culture was treated with herbal products or control drugs. The beginning CFU for the five-day-old stationary phase *B. henselae* culture was about 2×10^7 CFU/mL. At different time points of drug exposure (day 1, day 3, day 5, and day 7), portions of bacteria were removed, washed, and plated on Columbia blood agar for CFU counts. [2] The herbal product concentration used in

this experiment was 0.25% (*v/v*). Drug concentrations used in this experiment were based on their Cmax and were as follows: 0.2 µg/mL AZI, 2.4 µg/mL DOX, 10 µg/mL GEN, and 7.8 µg/mL RIF.

II. Evaluation of essential oil collection for activity against *B. henselae*

This part of the study was recently published: X. Ma, W. Shi, Y. Zhang. Essential Oils with High Activity against Stationary Phase *Bartonella henselae*. Antibiotics. 2019, 8(4), 246. https://doi.org/10.3390/antibiotics8040246

Screening the essential oil collection to identify drugs active against non-growing stationary phase *B. henselae*

Similarly, we adapted the SYBR Green I/PI viability assay developed previously [55] to identify essential oils with good activity against stationary phase *B. henselae*. As described above, we tested a panel of essential oils and their corresponding controls against a five-day-old stationary phase *B. henselae* culture in 96-well plates incubated for three days. For primary screens, all 149 essential oils were applied at two

concentrations, 0.5% (*v/v*) and 0.25% (*v/v*), respectively. Meanwhile, the currently used antibiotics for bartonellosis treatment such as AZI, DOX, GEN, MXF, and RIF were included as control drugs for comparison, as well as the previously identified FDA-approved drugs effective against stationary phase *B. henselae* including DAP, methylene blue, miconazole, and NIT [55] (Table 3). All these antibiotics were used at 20 μM. In the primary screens, after the three-day drug exposure, 32 of 149 essential oils in the collection were found to have good activity against stationary phase *B. henselae* both at the concentration of 0.5% (*v/v*) and 0.25% (*v/v*), and thus were selected as top hits. The top 32 active hits were chosen based on their lower percentage of viable cells remaining after essential oil treatment than that for the current antibiotics used to treat *Bartonella* infections, including AZI, DOX, GEN, MXF, and RIF. Similar to herbal products, some compounds in the essential oils can cause interference with the SYBR Green I/PI assay because of color and autofluorescence. Thus, we selected these 32 top hits for further validation by microscopic counting to confirm the SYBR Green I/PI plate reader results. The currently used antibiotics for bartonellosis

treatment and the identified FDA-approved drugs effective against *B. henselae* were also included as controls for comparison at 20 μM. DOX as a control drug showed mild activity against stationary phase *B. henselae* (residual viability above 26%) (Table 3). Other clinically used antibiotics to treat *Bartonella* infection including MXF, GEN, AZI, and RIF [30,63] showed relatively better activity (residual viability between 9% and 25%) against stationary phase *B. henselae* than DOX. FDA-approved drugs that we identified as effective against stationary phase *B. henselae* (DAP, methylene blue, miconazole, and NIT) had better activity (residual viability between 8% and 19%) than most of the five antibiotics mentioned above.

Table 4. Activity of top 32 essential oils active against stationary phase *B. henselae* [1]

Essential Oils and Control Drugs	Plant or Ingredients of Essential Oils	Residual Viability (%) after 0.5% EO or 20 μM Antibiotic Treatment		Residual Viability (%) after 0.25% EO Treatment	
		Plate Reader[2]	Microscope[3]	Plate Reader[2]	Microscope[3]
Drug free control		74%	74%		
DOX		26%	57%		
GEN		9%	35%		
MXF		22%	40%		
AZI		23%	67%		
RIF		25%	44%		
DAP		8%	18%		

Methylene Blue		16%	27%		
NIT		18%	50%		
Miconazole		19%	44%		
Frankincense	*Boswellia serrata*	5%	11%	6%	10%
Ylang ylang	*Cananga odorata*	5%	9%	8%	10%
Tangerine	*Citrus reticulata*	6%	6%	5%	12%
Bergamot	*Citrus bergamia*	6%	18%	10%	15%
Marjoram (sweet)	*Origanum majorana*	6%	13%	5%	15%
Cajeput	*Melaleuca cajeputi*	7%	21%	9%	21%
Lemon	*Citrus limonum*	7%	10%	4%	11%
Oregano	*Origanum vulgare hirtum*	7%	7%	7%	20%
Geranium bourbon	*Pelargonium graveolens*	8%	20%	11%	22%
Tea tree	*Melaleuca alternifolia*	8%	12%	5%	25%
Fir needle	*Abies siberica*	8%	25%	10%	26%
Stress relief	synergy blend of essential oils of bergamot, patchouli, sweet orange, ylang ylang, pink grapefruit, gurjum	8%	15%	6%	12%
Mountain savory (winter)	*Satureja montana*	8%	25%	21%	32%
Bandit	synergy blend of essential oils of clove, cinnamon, lemon, rosemary, eucalyptus	8%	8%	12%	20%
Cinnamon leaf	*Cinnamomum zeylanicum*	8%	35%	10%	25%
Citronella	*Cymbopogon winterianus*	8%	15%	12%	23%
Health shield	blend of cinnamon, clove, eucalyptus, lemon and rosemary oils	9%	18%	17%	20%
Spearmint	*Mentha spicata*	9%	9%	4%	20%
Ho wood	*Cinnamomum camphora*	9%	20%	11%	29%
Tic Tox aux huiles essentielles	blend of essential oils of savory, sage officinale, wild chamomile, clove, compact oregano, cinnamon and niaouli	11%	21%	14%	14%

Citrus blast	synergy blend of *Citrus sinesis, Citrus limonum, Citrus reticulata blanco var tangerina, Citrus bergamia, Citrus reticulata, Citrus clementina, Vanilla planifolia*	11%	13%	11%	30%
Elemi	*Canarium luzonicum*	12%	25%	14%	32%
Vetiver	*Vetiveria zizanoides*	12%	26%	8%	18%
Grapefruit	*Citrus paradisi*	12%	35%	11%	36%
Clove bud 1	*Eugenia caryophyllata*	13%	36%	9%	23%
Deep forest	synergy blend of *Abies sibirica ledeb, Abies alba, Pinus sylvestris, Cupressus sempervirens, Cedrus deodora*	13%	20%	12%	50%
Geranium	*Pelargonium asperum*	14%	23%	15%	20%
Clove bud 2	*Syzygium aromaticum L*	15%	15%	14%	18%
Oil of oregano	*Origanum vulgare hirtum*	15%	52%	19%	55%
Allspice	*Pimenta officinalis*	16%	35%	6%	30%
Cedarwood	*Cedrus deodora*	17%	53%	10%	23%
Cinnamon bark	*Cinnamomum zeylanicum*	18%	40%	13%	45%

[1] A five-day-old stationary phase *B. henselae* culture was treated with essential oils (0.5% or 0.25%) (*v/v*) or control drugs (20μM) for three days. [2] Residual viability was calculated according to the regression equation and the ratio of Green/Red fluorescence obtained by SYBR Green I/PI assay. [3] Residual viability was assayed by fluorescence microscope counting.

The microscopic counting aligned well with the plate reader results. Among the 32 top hits that had better activity (residual viability between 5% and 21%) against stationary phase *B. henselae* than most control antibiotics, the most active essential oils

were ylang-ylang, lemon, stress relief, health shield, Tic Tox aux huiles essentielles, geranium essential oil, clove bud, and cedarwood because of their remarkable activity at 0.25%, as shown by red (dead) cells in fluorescence microscope observation (Figure 3). Essential oils made from oregano ("oregano" and "oil of oregano") and cinnamon ("cinnamon leaf" and "cinnamon bark") were all shown to be highly active against stationary phase *B. henselae*, which have already been identified effective against stationary phase *B. burgdorferi* in our previous study [51]. Some essential oils extracted from plants of the same genus as oregano or cinnamon also exhibited good activity against stationary phase *B. henselae.* For example, ho wood, which was also extracted from *Cinnamomum* spp. as cinnamon essential oils were shown to be active. Marjoram (sweet), which was extracted from *Origanum* spp. as oregano essential oils were also active. In addition, essential oils extracted from *Citrus* spp. including tangerine, bergamot, lemon, and grapefruit all exhibited strong activity against stationary phase *B. henselae*, and the same for essential oils extracted from *Pelargonium* spp. (geranium bourbon and geranium essential oil) and *Melaleuca* spp. (cajeput and tea tree essential

oil). Many blended essential oils including “stress relief”, “bandit”, “health shield”, “Tic Tox”, “citrus blast”, and “deep forest” exhibited strong activity against stationary phase *B. henselae* because some of their components, such as clove, ylang ylang, lemon, bergamot, grapefruit, cinnamon, oregano, and fir needle were shown to be active as single essential oils. Control drugs including DOX and AZI exhibited poor activity against stationary phase *B. henselae* as shown by green (live) cells using fluorescence microscope observation. Other antibiotics including GEN, MXF, RIF, DAP, methylene blue, NIT, and miconazole showed better activity, while not as good as most of the 32 top hits (Figure 3).

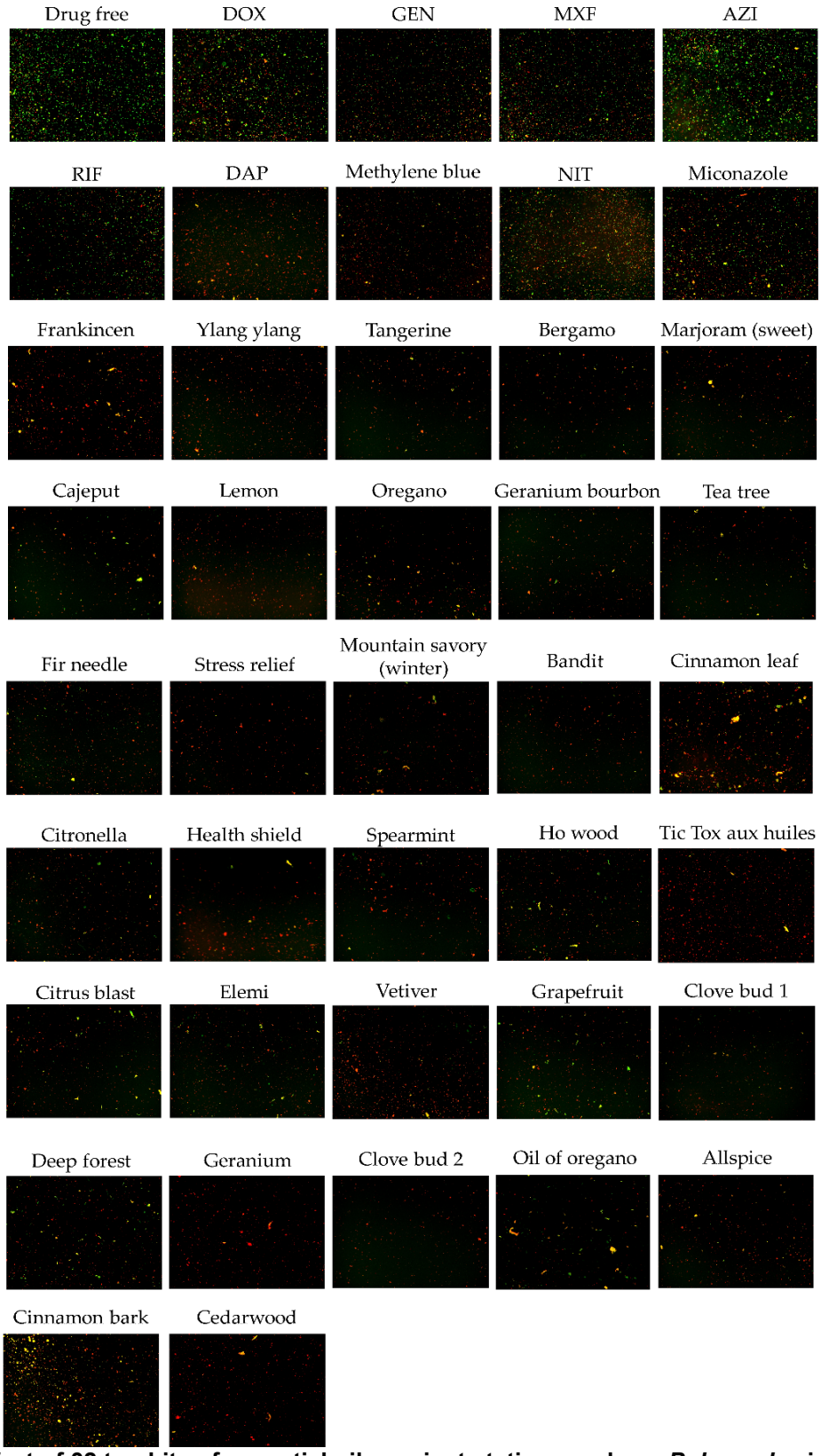

Figure 3. Effect of 32 top hits of essential oils against stationary phase *B. henselae* in comparison with control drugs. A five-day-old stationary phase *B. henselae* culture was treated with 0.25% (*v/v*)

essential oils or control antibiotics (20μM) for three days followed by SYBR Green I/PI viability assay and fluorescence microscopy (400 × magnification). Green cells represent live cells and red cells represent dead cells.

MIC determination of active hits in the essential oil collection

The essential oils listed above were active against the non-growing stationary phase *B. henselae* (Table 3 and Figure 3), and it was necessary to determine the MICs of these active drugs against log-phase growing *B. henselae*. The MICs of essential oils for *B. henselae* were determined by the standard microdilution method, as described in our previous study [55]. As shown in Table 4, cinnamon bark was the most active essential oil among these 32 hits, capable of inhibiting visible *B. henselae* proliferation at the lowest concentration of the essential oils tested (0.008%). The health shield, a blend of many active compounds against non-growing *B. henselae* was also highly active against growing *B. henselae*, which could inhibit *B. henselae* proliferation at a concentration of 0.008–0.016%. And the growth of *B. henselae* was efficiently suppressed by bandit, elemi, mountain savory (winter), cedarwood and two oregano essential oils at 0.016–0.032%, and by ylang-ylang, citronella, clove bud, geranium

bourbon, allspice, vetiver, cinnamon leaf and geranium essential oil at 0.032–0.063%. Other single essential oils including bergamot, cajeput, marjoram (sweet), fir needle, grapefruit as well as blends of essential oils including stress relief, citrus blast, and deep forest were also active with MIC values of 0.063–0.125%. *B. henselae* growing cells were also susceptible to spearmint, tangerine, tea tree, lemon, ho wood, frankincense, and Tic Tox aux huiles essentielles at a concentration of 0.125–0.25%, although at relatively higher concentrations than other oils tested.

Table 5. Minimum inhibitory concentrations (MICs) of top active essential oils against *B. henselae*

Essential Oils	Plant or Ingredients of Essential Oils	MIC (*v/v*)
Cinnamon bark	*Cinnamomum zeylanicum*	<0.008%
Health shield	blend of cinnamon, clove, eucalyptus, lemon and rosemary oils	0.008–0.016%
Bandit	synergy blend of essential oils of clove, cinnamon, lemon, rosemary, eucalyptus	0.016–0.032%
Oregano	*Origanum vulgare hirtum*	0.016–0.032%
Elemi	*Canarium luzonicum*	0.016–0.032%
Oil of oregano	*Origanum vulgare hirtum*	0.016–0.032%
Mountain savory (winter)	*Satureja montana*	0.016–0.032%
Cedarwood	*Cedrus deodora*	0.016–0.032%
Ylang ylang	*Cananga odorata*	0.032–0.063%
Citronella	*Cymbopogon winterianus*	0.032–0.063%
Clove bud 1	*Eugenia caryophyllata*	0.032–0.063%
Clove bud 2	*Syzygium aromaticum L*	0.032–0.063%
Geranium bourbon	*Pelargonium graveolens*	0.032–0.063%
Allspice	*Pimenta officinalis*	0.032–0.063%

Vetiver	*Vetiveria zizanoides*	0.032–0.063%
Cinnamon leaf	*Cinnamomum zeylanicum*	0.032–0.063%
Geranium	*Pelargonium asperum*	0.032–0.063%
Stress relief	synergy blend of essential oils of bergamot, patchouli, sweet orange, ylang ylang, pink grapefruit, gurjum	0.063–0.125%
Bergamot	*Citrus bergamia*	0.063–0.125%
Cajeput	*Melaleuca cajeputi*	0.063–0.125%
Marjoram (sweet)	*Origanum majorana*	0.063–0.125%
Citrus blast	synergy blend of essential oils of bergamot, patchouli, sweet orange, ylang ylang, pink grapefruit, gurjum	0.063–0.125%
Deep forest	synergy blend of *Abies sibirica ledeb, Abies alba, Pinus sylvestris, Cupressus sempervirens, Cedrus deodora*	0.063–0.125%
Fir needle	*Abies siberica*	0.063–0.125%
Grapefruit	*Citrus paradisi*	0.063–0.125%
Spearmint	*Mentha spicata*	0.125–0.25%
Tangerine	*Citrus reticulata*	0.125–0.25%
Tea tree	*Melaleuca alternifolia*	0.125–0.25%
Lemon	*Citrus limonum*	0.125–0.25%
Ho wood	*Cinnamomum camphora*	0.125–0.25%
Frankincense	*Boswellia serrata*	0.125–0.25%
Tic Tox aux huiles essentielles	blend of essential oils of savory, sage officinale, wild chamomile, clove, compact oregano, cinnamon and niaouli	0.125–0.25%

Time-kill curves of active hits in the essential oil collection

Having obtained 32 top hits by primary screens, we performed a time-kill drug exposure assay against a five-day-old stationary phase *B. henselae* culture at a lower concentration of these active essential oils. Here we just selected single essential oil samples for a drug exposure assay in order to better evaluate and compare the activity of antimicrobial components among different essential oils. All selected 25 essential oils

were applied at 0.032% (*v/v*), respectively. Clinically used antibiotics and the previously identified effective FDA-approved drugs against *B. henselae* were used at their Cmax as controls. As shown in Table 5 and Figure 4b & 4c, oregano, cinnamon bark, and mountain savory (winter) were the most active essential oils that rapidly killed *B. henselae* with no detectable CFU after one-day exposure. Other active hits, including clove bud 2, allspice, geranium, and cinnamon leaf could eradicate *B. henselae* cells without viable cells being recovered after a three-day drug exposure. Geranium bourbon and clove bud 1 also showed excellent activity which could kill all bacteria by day 5, followed by elemi, vetiver, citronella and ylang ylang that eradicated all *B. henselae* cells by day 7.

As shown in Table 5, grapefruit, tangerine, bergamot, fir needle, frankincense and ho wood were also quite active, reducing 5 log10 CFU/mL after a seven-day exposure. Lemon, as well as marjoram (sweet) also had the capability of killing stationary phase *B. henselae* and reduced the bacterial count by approximately 3 log10 CFU/mL in seven days. However, cajeput, tea tree, cedarwood, and spearmint showed poor activity.

Compared with the drug-free control, as shown in Figure 4a, some clinically used antibiotics for *Bartonella* treatment, such as AZI and DOX, had poor activity in killing *B. henselae*, achieving approximately 1 log10 CFU/mL decrease after the seven-day drug exposure. GEN and RIF showed better activity than AZI and DOX when used at their Cmax, which could eradicate all *B. henselae* cells respectively by day 3 and day 7. Other FDA-approved drugs effective against *B. henselae* including DAP and methylene blue had good activity that led to the eradication of *B. henselae* cells after a one-day or five-day exposure, respectively, while miconazole did not kill all *B. henselae* cells by day 7 when used at Cmax.

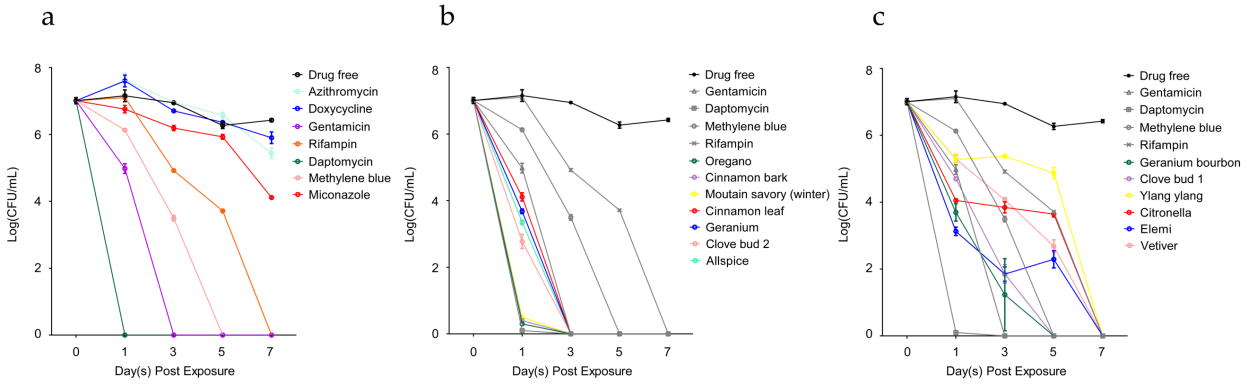

Figure 4. Time-kill curves of active essential oil treatment against stationary phase *B. henselae* in comparison with control drugs. (a) Time-kill curves for control antibiotic treatment. **(b–c)** Time-kill curves for essential oil treatment. Drug-free control, DAP, GEN, methylene blue, and RIF treatment were the same

among a, b, and c. The essential oils or control antibiotics were added to a five-day-old stationary phase culture respectively at time point 0, and at different times of drug exposure (day 1, day 3, day 5, and day 7), portions of bacteria were removed and washed and plated on Columbia blood agar plates for CFU counts. The essential oil concentration used in this experiment was 0.032% (*v/v*). Drug concentrations used in this experiment were based on their Cmax and were as follows: 0.2 μg/mL AZI, 2.4 μg/mL DOX, 10 μg/mL GEN, 7.8 μg/mL RIF, 60 μg/mL DAP, 2.9 μg/mL methylene blue, and 6.3 μg/mL miconazole.

Table 6. Drug exposure assay of top active essential oils against *B. henselae* stationary phase culture [1]

Essential Oils and Control Drugs[2]	CFU/mL after Drug Exposure			
	1 Day	3 Day	5 Day	7 Day
Drug free control	$1.50 \pm 0.53 \times 10^7$	$8.83 \pm 0.29 \times 10^6$	$1.88 \pm 0.40 \times 10^6$	$2.67 \pm 0.29 \times 10^6$
DOX	$4.17 \pm 1.44 \times 10^7$	$5.07 \pm 0.38 \times 10^6$	$2.30 \pm 0.10 \times 10^6$	$8.33 \pm 2.89 \times 10^5$
AZI	$4.50 \pm 2.00 \times 10^7$	$9.17 \pm 0.29 \times 10^6$	$3.80 \pm 0.72 \times 10^6$	$2.83 \pm 1.04 \times 10^5$
GEN	$9.83 \pm 2.93 \times 10^4$	0	0	0
RIF	$1.27 \pm 0.15 \times 10^7$	$8.33 \pm 0.76 \times 10^4$	$5.17 \pm 0.29 \times 10^3$	0
DAP	0	0	0	0
Methylene blue	$1.35 \pm 0.13 \times 10^6$	$3.17 \pm 0.58 \times 10^3$	0	0
Miconazole	$5.83 \pm 1.53 \times 10^6$	$1.57 \pm 0.28 \times 10^6$	$8.50 \pm 1.32 \times 10^5$	$1.30 \pm 0.10 \times 10^4$
Oregano	0	0	0	0
Cinnamon bark	0	0	0	0
Mountain savory (winter)	0	0	0	0
Clove bud 2	$6.50 \pm 3.46 \times 10^2$	0	0	0
Allspice	$2.27 \pm 0.33 \times 10^3$	0	0	0
Geranium	$4.83 \pm 0.76 \times 10^3$	0	0	0
Cinnamon leaf	$1.33 \pm 0.35 \times 10^4$	0	0	0
Geranium bourbon	$5.50 \pm 2.65 \times 10^3$	$5.00 \pm 5.00 \times 10$	0	0
Clove bud 1	$5.00 \pm 0.00 \times 10^4$	$8.33 \pm 5.77 \times 10$	0	0
Elemi	$1.38 \pm 0.42 \times 10^3$	$5.00 \pm 5.00 \times 10$	$2.17 \pm 1.04 \times 10^2$	0
Vetiver	$2.00 \pm 0.50 \times 10^5$	$1.18 \pm 0.19 \times 10^4$	$5.17 \pm 2.47 \times 10^2$	0
Citronella	$1.13 \pm 0.12 \times 10^4$	$7.33 \pm 2.84 \times 10^3$	$4.50 \pm 0.87 \times 10^3$	0
Ylang ylang	$2.00 \pm 0.87 \times 10^5$	$2.38 \pm 0.19 \times 10^5$	$7.83 \pm 3.01 \times 10^4$	0
Grapefruit	$1.02 \pm 0.19 \times 10^4$	$3.17 \pm 1.89 \times 10^4$	$5.33 \pm 1.26 \times 10^3$	$6.67 \pm 5.77 \times 10$
Tangerine	$3.17 \pm 0.29 \times 10^4$	$2.08 \pm 0.58 \times 10^4$	$4.50 \pm 2.29 \times 10^3$	$6.67 \pm 5.77 \times 10$
Bergamot	$8.17 \pm 2.25 \times 10^3$	$2.62 \pm 0.35 \times 10^4$	$6.83 \pm 0.76 \times 10^3$	$1.67 \pm 0.58 \times 10^2$

Fir needle	$4.17 \pm 1.61 \times 10^3$	$2.32 \pm 0.41 \times 10^4$	$1.10 \pm 0.13 \times 10^4$	$1.67 \pm 0.58 \times 10^2$
Frankincense	$1.35 \pm 0.22 \times 10^5$	$8.17 \pm 1.53 \times 10^5$	$1.48 \pm 0.29 \times 10^6$	$1.83 \pm 0.76 \times 10^2$
Ho wood	$5.00 \pm 0.50 \times 10^6$	$7.50 \pm 2.65 \times 10^5$	$1.37 \pm 0.28 \times 10^5$	$4.17 \pm 1.44 \times 10^2$
Lemon	$3.17 \pm 1.15 \times 10^4$	$1.03 \pm 0.28 \times 10^5$	$8.67 \pm 0.76 \times 10^4$	$4.33 \pm 2.31 \times 10^3$
Marjoram (Sweet)	$2.17 \pm 1.53 \times 10^5$	$2.13 \pm 0.28 \times 10^6$	$2.22 \pm 0.25 \times 10^6$	$7.50 \pm 1.32 \times 10^3$
Cajeput	$2.50 \pm 0.87 \times 10^6$	$9.43 \pm 0.40 \times 10^6$	$3.20 \pm 0.26 \times 10^6$	$1.62 \pm 0.25 \times 10^5$
Tea tree	$8.00 \pm 2.18 \times 10^5$	$9.33 \pm 0.29 \times 10^6$	$3.97 \pm 0.45 \times 10^6$	$3.17 \pm 0.76 \times 10^6$
Cedarwood	$2.33 \pm 2.31 \times 10^5$	$2.73 \pm 0.33 \times 10^6$	$3.40 \pm 0.36 \times 10^6$	$3.52 \pm 0.18 \times 10^6$
Spearmint	$4.33 \pm 1.26 \times 10^5$	$9.17 \pm 0.29 \times 10^6$	$3.67 \pm 0.58 \times 10^6$	$3.68 \pm 0.38 \times 10^6$

[1] A five-day-old stationary phase *B. henselae* culture was treated with essential oils or control drugs. The beginning CFU for the five-day-old stationary phase *B. henselae* culture was about 1×10^7 CFU/mL. At different time points of drug exposure (day 1, day 3, day 5, and day 7), portions of bacteria were removed, washed, and plated on Columbia blood agar for CFU counts. [2] The essential oil concentration used in this experiment was 0.032% (*v/v*). Drug concentrations used in this experiment were based on their Cmax and were as follows: 2.4 μg/mL DOX, 0.2 μg/mL AZI, 10 μg/mL GEN, 7.8 μg/mL RIF, 60 μg/mL DAP, 2.9 μg/mL methylene blue, and 6.3 μg/mL miconazole.

Carvacrol and cinnamaldehyde as active ingredient of essential oils are highly potent against stationary phase *B. henselae*

Our previous studies have identified two components, carvacrol and cinnamaldehyde, as highly potent active ingredients of oregano and cinnamon bark essential oils, respectively, which were effective against *B. burgdorferi* [51,52]. As shown above, oregano and cinnamon bark essential oils were also highly active to kill *B. henselae*, so we tested carvacrol and cinnamaldehyde, two major constituents of

these two active essential oils, for their antimicrobial activity against *B. henselae*. Carvacrol and cinnamaldehyde were applied at two concentrations, 0.01% (*v/v*) and 0.005% (*v/v*), respectively, for the drug exposure assay against a five-day-old stationary phase *B. henselae* culture. Clinically used antibiotics and the previously identified effective FDA-approved drugs against *B. henselae* were used at their Cmax as controls. As shown in Figure 5, 0.01% carvacrol could eradicate *B. henselae* cells without viable cells being recovered after a five-day drug exposure. Additionally, 0.005% carvacrol led to 2 log10 CFU/mL reduction after a seven-day exposure. Cinnamaldehyde was especially active such that it rapidly killed all stationary phase *B. henselae* cells with no detectable CFU after one-day exposure when used at the concentration of 0.01%, and 0.005% cinnamaldehyde could also eradicate all *B. henselae* cells after a three-day exposure.

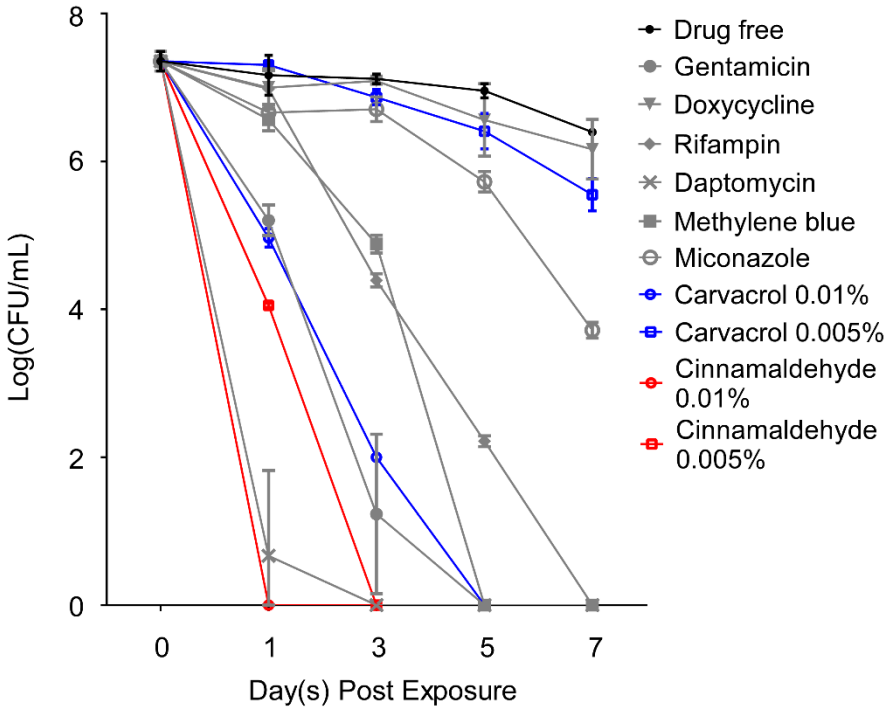

Figure 5. Time-kill curves of carvacrol and cinnamaldehyde treatment against stationary phase *B. henselae* in comparison with control drugs. Carvacrol, cinnamaldehyde or control antibiotics were added to a five-day-old stationary phase culture respectively at time point 0, and at different time points of drug exposure (day 1, day 3, day 5, and day 7), portions of bacteria were removed and washed and plated on Columbia blood agar for CFU counts. The concentrations of carvacrol and cinnamaldehyde used were 0.01% (*v/v*) and 0.005% (*v/v*). Drug concentrations used in this experiment were based on their Cmax and were as follows: 2.4 μg/mL DOX, 10 μg/mL GEN, 7.8 μg/mL RIF, 60 μg/mL DAP, 2.9 μg/mL methylene blue, and 6.3 μg/mL miconazole.

DISCUSSION

Many chronic persistent bacterial infections pose challenges for clinical treatment, including the chronic *B. henselae* infection, which is thought to be mainly due to persister bacteria that are not effectively killed by the currently used antibiotics [23]. To address this problem, we successfully applied the SYBR Green I/PI viability assay for the high-throughput screen of a herbal product collection and an essential oil collection for activity against stationary phase *B. henselae* as a model of persister drug screens. We identified some herbal products that had high activity at 1% (*v/v*) concentration, including extracts of *Juglans nigra*, *Cryptolepis sanguinolenta*, *Polygonum cuspidatum*, *Scutellaria baicalensis*, and *Scutellaria barbata*. Among these top hits, three herbal product extracts could eradicate all stationary *B. henselae* cells without CFU being detected within a seven-day drug exposure at a low concentration of 0.25%, including Cryptolepis (*Cryptolepis sanguinolenta*) 30%, 60%, 90% alcohol extracts, black walnut (*Juglans nigra*) 60%, 90% alcohol extracts, and Japanese knotweed (*Polygonum cuspidatum*) extracts. In addition, we identified 32 essential oils at 0.25% (*v/v*)

concentration which had good activity against stationary phase *B. henselae*. These included four essential oils extracted from plants of genus *Citrus* (tangerine, bergamot, lemon and grapefruit), three from *Origanum* (two oregano essential oils and marjoram), three from *Cinnamomum* (cinnamon bark, cinnamon leaf and ho wood), two from *Pelargonium* (geranium bourbon and geranium essential oil) and two from *Melaleuca* (cajeput and tea tree). Among these 32 top hits, thirteen single essential oils could effectively kill all stationary *B. henselae* cells without CFU recovered within the seven-day drug exposure even at a low concentration of 0.032% (*v/v*), where the essential oils of oregano, cinnamon bark, and mountain savory (winter) were the most active ones that eradicated all bacteria after a one-day exposure. Some essential oils that showed activity by primary screens exhibited poor activity in drug exposure assay, partly due to the volatility of essential oils during such a long incubation period. Carvacrol and cinnamaldehyde, two active ingredients of effective essential oils, oregano and cinnamon bark, respectively, were shown to be extremely active against stationary phase *B. henselae* being able to eradicate all bacterial cells within a seven-day drug

exposure, even at a very low concentration ≤ 0.01% (*v/v*). The MIC determination of these active hits showed they were not only active against stationary phase *B. henselae* but also effective in inhibiting the growth of log phase *B. henselae*, especially the essential oil of cinnamon bark.

The observation that oils from different plant species of the same genus all possess antimicrobial ability could serve as a guide in future studies to obtain more active hits and decode the antimicrobial mechanism. Here, we identified *Citrus*, *Cinnamomum*, *Origanum*, *Pelargonium*, and *Melaleuca* as potential genera that might include more plants the oils of which are active against *B. henselae*. *Citrus* plants constitute one of the most valuable and important sources of essential oil served in food processing and medical use. *Citrus limonum* essential oil was reported to have antimicrobial activities with preservative effect against *Listeria monocytogenes* inoculated in minced beef meat [64], and remarkable miticidal activity *in vitro* and *in vivo* applications against sarcoptic mange in rabbits [65]. Besides, the essential oil from *Citrus limetta* Risso peels could alleviate skin inflammation, both tested *in vitro* and *in vivo* [66], while the essential oil

from *Citrus aurantium* L. var. *amara* Engl also had an anti-inflammatory effect [67]. *Citrus* leaf extract was reported to reduce blood pressure and vascular damage in repeatedly heated palm oil diet-induced hypertensive rats [68]. These studies indicated *Citrus* plants could serve in different health care treatments including antimicrobial function. Our study was the first to identify their activity against *B. henselae*.

Previous studies have shown that oregano oil has antibacterial activity against three Gram-positive and two Gram-negative bacteria of their growing log phase [69]. It was also reported to be highly effective against stationary phase *B. burgdorferi* [51]. Cinnamon, clove bud, and allspice were well-known as flavors for food processing, while they were both found to have excellent activity against *B. burgdorferi* stationary phase cells *in vitro*, even better than the persister drug daptomycin [51,52]. Allspice was also known to have antibacterial activities on many organisms [70]. Here, for the first time, we identified essential oils of oregano, cinnamon, clove bud and allspice as having highly potent activities against both the log phase and stationary phase *B. henselae*. It is interesting to note that the high activity of these common essential oils against both

Borrelia and *Bartonella*, such as oregano, cinnamon bark, and clove bud, indicated that they had the potential to be active against both *Borrelia* and *Bartonella* persistent infections, which clinically may be present as coinfections [16]. However, it is also worth noting that some other essential oils including frankincense, ylang-ylang, fir needle, mountain savory (winter), elemi, and vetiver, are preferentially more active against *B. henselae.* This suggests preferential activity of some essential oils against different bacterial species that possess different cell surface structures, efflux, and physiology.

Other essential oils identified as effective in our study have also been proved to have good biological activities by previous studies. It was reported that frankincense and geranium essential oils could suppress tumor progression through the regulation of the AMPK/mTOR pathway in breast cancer [71]. Geranium essential oil could eradicate enterococcal biofilm at a concentration of 150 mg/mL without bacteria developing resistance to it; thus it could be a possible alternative to other antimicrobials during endodontic procedures [72]. Frankincense was reported to have anti-inflammatory and antibacterial effects [73]. Mountain savory has been proved to be highly active against

methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella typhimurium* and *L. monocytogenes* [74-76]. According to previous studies, fir honeydew honey had strong antimicrobial activity against *S. aureus, A. baumannii, P. aeruginosa*, *E. coli* and many kinds of fungi [77-79]. Ylang-ylang products have a wide variety of bioactivities including antimicrobial, antibiofilm, anti-inflammatory, anti-vector, insect-repellent and so on, demonstrating it to be a useful plant to agriculture and medicine [80]. Citronella was reported to have antifungal and antibiofilm activity as well as antimicrobial activity against *Staphylococcus* [81,82]. Extracts from *Canarium* were proved to be active against MRSA and *P. aeruginosa* [83]. Essential oils of vetiver were active against *S. aureus* and showed good antifungal and cytotoxic activities [84,85]. Here, for the first time, we identified the remarkable activity of these essential oils active against both log phase and stationary phase *B. henselae*.

Similarly, plant species of which the herbal extracts we found to be active against *B. henselae* have also been reported to have various biological activities by previous studies. Different parts of various species from genus *Juglans* have shown pain-

relieving, antioxidant, antibacterial, antifungal and antitumor activities [86-88]. In particular, different *Juglans regia* cultivars and one active ingredient, juglone, showed excellent antifungal activity and *Juglans nigra* exhibited both bacteriostatic activity and bactericidal activity against *Borrelia* based on *in vitro* studies [58,89]. Previous studies have profiled the phytochemicals of *Juglans* plants, including different types of steroids, flavonoid C-glycoside, flavones, essential oil component, tannins and miscellaneous [90]. Many active ingredients of *Juglans* with potential importance to human health were identified, such as juglone, phenolic acids, flavonoids and catechins, and the safety as well as efficacy of these compounds were systematically assessed, concluded to be with great benefit [91-95]. A study comparing leaf essential oils of *J. regia* and *J. nigra* further showed *J. nigra* leaf oil was less phytotoxic [96]. *Cryptolepis sanguinolenta* and its constituents were reported to have many excellent biological activities including antibacterial, antifungal, anti-inflammatory, anticancer, antimalaria and anti-amoebic properties [59,97-101]. A systematic review has assessed the phytochemistry and pharmacology of *Cryptolepis sanguinolenta* and concluded that although there have

been data pointing to the toxic potential of this plant, it is still a promising source of potential agent(s) that can aid in many disease therapies [102]. Among constituents and secondary metabolites of the plant identified with antimicrobial activity, an alkaloid called cryptolepine was the most well-studied and considered to be the most important active component. Cryptolepine was reported to have a lytic effect on *S. aureus* as seen in SEM photomicrographs which led to altered cell morphology and could intercalate into DNA at cytosine-cytosine sites or inhibited the activity of topoisomerase causing DNA damage [103-105]. Future studies are needed to decode more specific antibacterial mechanisms of cryptolepine as well as other active ingredients when used against bacterial pathogens including *B. henselae*. *Polygonum cuspidatum* has been documented to have antibacterial effects against *Vibrio vulnificus* [106], *Streptococcus mutans* [107] and streptococcus associated biofilms [108]. Its constituents have also been shown to have antimicrobial, anti-tumor, anti-inflammatory, neuroprotective, and cardioprotective effects [109-113]. One of the most active constituents is a polyphenol called resveratrol, which was reported to be active against log phase *Borrelia*

burgdorferi and *Borrelia garinii* by *in vitro* testing [58]. In addition, another active constituent called emodin (6-methyl-1,3,8-trihydroxyanthraquinone) has been shown to have activity against stationary phase *Borrelia burgdorferi* cells [114]. There is a study unraveling the action mechanism of *Polygonum cuspidatum* using a network pharmacology approach, which suggested that polydatin might be the critical active component of *Polygonum cuspidatum* [115]. *Polygonum cuspidatum* has been found to have minimal toxicity in animal and human studies. It is reported that gastrointestinal upset and diarrhea can occur as a side effect but can resolve with lowering of doses or cessation of therapy [116]. Our study is the first to identify the antimicrobial activity of extracts from *Juglans nigra*, *Cryptolepis sanguinolenta*, and *Polygonum cuspidatum* against stationary phase *B. henselae.* In addition, considering the possibility of coinfection caused by *Borrelia burgdorferi* and *B. henselae* during Lyme disease development, the overlap of active herbal products against both *B. henselae* identified in our study and against *B. burgdorferi* according to previous studies, including *Juglans*

nigra, *Cryptolepis sanguinolenta*, and *Polygonum cuspidatum*, should provide a promising strategy for better treatment of coinfections with both pathogens [53].

According to our data in Table 1, Table 3, Table 5, and Figure 1-5, some clinically used antibiotics for treating *Bartonella*-associated infections including AZI and DOX showed weak activity in eradicating stationary phase *B. henselae* cells, which coincides with the reported discrepant antibiotic efficacies between *in vitro* MIC data and clinical data from patients [30]. The poor activities of current clinically used antibiotics against stationary phase *B. henselae* as shown in our study could partly explain the treatment failure due to persistent infection. This discrepancy may be partly due to the antibacterial mechanisms of these antibiotics. DOX inhibits bacterial protein synthesis by binding to their 30S ribosomal subunit [117]. AZI could also inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit, and thus prevent bacteria from growing [118]. Although these antibiotics all target growing bacteria, they are not very effective at killing non-growing stationary phase *B. henselae*, and thus could lead to the treatment failure against persistent and chronic infections. However, the herbal extracts

and essential oils should be promising candidates to treat persistent *B. henselae* because of their abundant content of active organics including steroids, flavones, tannins and so on. These organics are lipophilic and could target the bacterial cell membrane, which is an important target of persister drugs like pyrazinamide [119] and daptomycin [120], especially when persistent bacterial cells aggregated together (Figure 1 and Figure 3) and protected cells located towards the center of the aggregates from attack by antimicrobial agents. However, it should also be noted that the high lipophilicity of essential oils might cause *B. henselae* cells or biofilm structures to be dissolved, leading to a reduction of the dead cell number and as a result, the residual viability percentage by the SYBR Green I/PI assay might be misinterpreted. Furthermore, some nonpolar essential oils are DMSO-insoluble, which could just form emulsions. They are hard to be distributed evenly in the bacterial culture, leading to different concentrations in different local areas and non-uniform antibacterial effects. It is also worth noting that as DMSO could permeabilize the bacterial membranes, it could

increase the antibacterial effect of the herbal products and essential oils by increasing the bacterial susceptibility to lipophilic compounds.

Despite the promising findings of the herbal extracts and essential oils active against *B. henselae*, future studies are needed to identify the active ingredients of these herbs and to decode their specific antimicrobial mechanisms. Here we just tested two active ingredients, carvacrol, and cinnamaldehyde, which showed excellent ability to eradicate stationary phase *B. henselae* even at a much lower concentration (≤ 0.01%) than would have been expected from the studies with the related essential oil samples. It is still worth noticing that according to the concentration of the original stock, 0.005% carvacrol or cinnamaldehyde was approximately equal to 50 μg/mL, which was a concentration similar to the Cmax of some clinically used antibiotics, such as DAP. Thus, on a weight basis, the antimicrobial activity of carvacrol and cinnamaldehyde against stationary phase *B. henselae* was comparable to that of effective FDA-approved antibiotics, including GEN, RIF, DAP, and methylene blue, which could eradicate all *B. henselae* cells within the seven-day drug exposure when used at their Cmax. Although

if compared on a molar basis, as the molecular weight of carvacrol and cinnamaldehyde are lower than that of most antibiotics mentioned above, these ingredients are not as effective as currently used antibiotics. Therefore, it would be of interest to test compounds like juglone, cryptolepine, and resveratrol, which are known active components of black walnut, cryptolepis, and Japanese knotweed herbs on *B. henselae* in future studies. Some previous studies have identified the main ingredients of some active essential oils such as mountain savory, thyme, lemongrass, limette, and cumin, including geranial, β-pinene, thymol, γ-terpinene, citronellal and so on [121]. Also, different parts of the plant might have different antimicrobial activities because of varying concentrations of the active compounds they contained, and different solvents used to extract the compounds could also significantly affect their activity. Therefore, the antimicrobial activity and pharmacokinetic profiles of active components should be studied thoroughly in the future, as well as the optimal extraction strategy to obtain maximum effective ingredients using minimum amount of plant materials, in order to better determine the utility and practicality of these active herbs. For example, based on

GC-MS profiles of active herbal products or essential oils, we should obtain a list of potential active ingredients. Future studies are needed to identify the activity of these ingredients on growing *B. henselae* or persistent *B. henselae* models compared with currently used antibiotics, both *in vitro* and *in vivo*. Pharmacokinetic studies are needed to measure the Cmax and half-life, as well as the potential toxicity of these active ingredients to determine whether they could obtain the expected concentration in the human body, while being free of toxic side effects.

Another promising strategy for developing a more effective treatment for *Bartonella* infections is the drug combination of active ingredients of herbal products or essential oils with antibiotics to avoid resistance development and improve the efficacy of the treatment. Future studies are needed to evaluate drug combinations of two or more newly identified active herbal ingredients with current clinically used drugs, in order to better target diverse bacterial populations of different phases or forms that can happen in the host as indicated by the Yin-Yang model [23]. There were some previous studies of evaluating the antimicrobial activity of combined essential oil samples against

multidrug resistance (MDR) *E. coli*, *K. pneumoniae*, MRSA, *S. epidermidis*, *Propionibacterium acnes*, as well as airborne bacteria and fungi in hospital rooms, and some of the essential oil combinations did have better activity than when used alone [122–124].

Regarding the phenomenon of bacterial persistence, many pathogenic bacteria are able to predominantly colonize body surfaces and tissues in multicellular aggregates such as biofilms [125, 126]. Formation of these sessile communities, which limits the access of the immune system components and drugs, combined with the persisters' inherent resistance to antimicrobial agents, is at the root of many persistent and chronic bacterial infections or frequent relapse after treatment. As biofilms were suggested to play an important role in chronic *B. henselae* infections, future studies are required to test active drug candidates and drug combinations on *B. henselae* biofilm models to further address the problem of bacterial persistence.

In this study, we identified a range of herbal products and essential oils with high activity against stationary phase *B. henselae in vitro*. Because *B. henselae* can reside

and propagate inside erythrocytes and/or endothelial cells in humans and animals [13,127], which could provide a shelter that protects them from the host immune responses and exposure to antibiotics, future studies are needed to evaluate the activities of selected herbal products and essential oils against intracellular *B. henselae*. The active ingredients of many effective herbal products and essential oils remain unknown, and it will take substantial effort to characterize and identify the active components, which is beyond the scope of the current study. These should be studied thoroughly in the future in order to identify the active components, decode the antimicrobial mechanism, and further evaluate their activity *in vivo*. We are fully aware that while the number of active components of herbal products and essential oils may be subject to variations from different batches or sources, just like any natural products, this should not change the overall findings or conclusions of the study. As far as we know, the effective concentration on a weight basis of two highly active ingredients, carvacrol, and cinnamaldehyde, was comparable to the Cmax of some antibiotics, indicating they could serve as promising drug candidates that may achieve efficacy

when used *in vivo*, which requires further pharmacokinetic studies. Many previous studies have demonstrated the activity of essential oils *in vivo*. For example, *Citrus limon* essential oil was proved to have high miticidal activity in rabbit models [65]. The safety and activity of the essential oil from *Citrus limetta* Risso peels for alleviating skin inflammation was identified using both rabbit and mouse models [66], and frankincense essential oil was shown to modulate tumor growth in a xenograft mouse model [71]. Further validation using appropriate animal models of *Bartonella* infections is required to assess the safety and efficacy of identified active herbal medicines and essential oils or their active components *in vivo*. Our study was performed with *B. henselae* and future studies are needed to test if the findings here apply to other *B. henselae* strains and also closely related pathogenic *Bartonella* species, such as *B. quintana* and *B. bacilliformis*.

REFERENCES

1. Brenner, D.J.; O'connor, S.P.; Winkler, H.H.; Steigerwalt, A.G. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of Bartonella quintana comb. nov., *Bartonella vinsonii comb. nov.*, *Bartonella henselae comb. nov.*, and *Bartonella elizabethae comb. nov.*, and to remove the family *Bartonellaceae* from the order *Rickettsiales*. *Int. J. Syst. Bacteriol*. **1993**, 43, 777-786.
2. Peters, D.; Wigand, R. Bartonellaceae. *Bacteriol. Rev*. **1955**, 19, 150-159.
3. Okaro, U.; Addisu, A.; Casanas, B.; Anderson, B. *Bartonella* Species, an Emerging Cause of Blood-Culture-Negative Endocarditis. *Clin. Microbiol. Rev*. **2017**, 30, 709-746.
4. Jacomo, V.; Kelly, P.J.; Raoult, D. Natural history of Bartonella infections (an exception to Koch's postulate). *Clin Diagn Lab Immunol*. **2002**, 9, 8-18.
5. Mullins, K.E.; Hang, J.; Clifford, R.J.; Onmus-Leone, F.; Yang, Y.; Jiang, J.; Leguia, M.; Kasper, M.R.; Maguina, C.; Lesho, E.P. et al. Whole-Genome Analysis of Bartonella ancashensis, a Novel Pathogen Causing Verruga Peruana, Rural Ancash Region, Peru. *Emerg Infect Dis*. **2017**, 23, 430-438.
6. Breitschwerdt, E.B. Bartonellosis, One Health and all creatures great and small. *Vet. Dermatol*. **2017**, 28, 96-e21.
7. Mosepele, M.; Mazo, D.; Cohn, J. Bartonella infection in immunocompromised hosts: immunology of vascular infection and vasoproliferation. *Clin Dev Immunol*. **2012**, 2012, 612809.
8. Hong, J.; Li, Y.; Hua, X.; Bai, Y.; Wang, C.; Zhu, C.; Du, Y.; Yang, Z.; Yuan, C. Lymphatic Circulation Disseminates *Bartonella* Infection Into Bloodstream. *J. Infect. Dis*. **2017**, 215, 303-311.

9. Rolain, J.M.; La Scola, B.; Liang, Z.; Davoust, B.; Raoult, D. Immunofluorescent detection of intraerythrocytic *Bartonella henselae* in naturally infected cats. *J. Clin. Microbiol*. **2001**, 39, 2978-2980.
10. Karem, K.L.; Paddock, C.D.; Regnery, R.L. Bartonella henselae, B. quintana, and B. bacilliformis: historical pathogens of emerging significance. *Microbes Infect*. **2000**, 2, 1193-1205.
11. Yuan, C.; Zhu, C.; Wu, Y.; Pan, X.; Hua, X. Bacteriological and molecular identification of Bartonella species in cats from different regions of China. *PLoS Negl Trop Dis*. **2011**, 5, e1301.
12. Klotz, S.A.; Ianas, V.; Elliott, S.P. Cat-scratch Disease. *Am Fam Physician*. **2011**, 83, 152-155.
13. Pulliainen, A.T.; Dehio, C. Persistence of Bartonella spp. stealth pathogens: from subclinical infections to vasoproliferative tumor formation. *FEMS Microbiol Rev*. **2012**, 36, 563-599.
14. Deng, H.; Pang, Q.; Xia, H.; Le Rhun, D.; Le Naour, E.; Yang, C.; Vayssier-Taussat, M.; Zhao, B. Identification and functional analysis of invasion associated locus B (IalB) in Bartonella species. *Microb Pathog*. **2016**, 98, 171-177.
15. Florin, T.A.; Zaoutis, T.E.; Zaoutis, L.B. Beyond cat scratch disease: widening spectrum of Bartonella henselae infection. *Pediatrics*. **2008**, 121, e1413-1425.
16. Eskow, E.; Rao, R.V.; Mordechai, E. Concurrent infection of the central nervous system by Borrelia burgdorferi and Bartonella henselae: evidence for a novel tick-borne disease complex. *Arch Neurol*. **2001**, 58, 1357-1363.
17. Chomel, B.B.; Boulouis, H.J.; Maruyama, S.; Breitschwerdt, E.B. *Bartonella* spp. in pets and effect on human health. *Emerg. Infect. Dis*. **2006**, 12, 389-394.
18. Gutierrez, R.; Vayssier-Taussat, M.; Buffet, J.P.; Harrus, S. Guidelines for the Isolation, Molecular Detection, and Characterization of *Bartonella* Species. *Vector. Borne.*

Zoonotic. Dis. **2017**, 17, 42-50.

19. Angelakis, E.; Raoult, D. Pathogenicity and treatment of *Bartonella* infections. *Int. J. Antimicrob. Agents*. **2014**, 44, 16-25.
20. Biswas, S.; Rolain, J.M. *Bartonella* infection: treatment and drug resistance. *Future. Microbiol*. **2010**, 5, 1719-1731.
21. Prutsky, G.; Domecq, J.P.; Mori, L.; Bebko, S.; Matzumura, M.; Sabouni, A.; Shahrour, A.; Erwin, P.J.; Boyce, T.G.; Montori, V.M. et al. Treatment outcomes of human bartonellosis: a systematic review and meta-analysis. *Int J Infect Dis*. **2013**, 17, e811-819.
22. Costerton, J.W.; Lewandowski, Z.; Caldwell, D.E.; Korber, D.R.; Lappin-Scott, H.M. Microbial biofilms. *Annu Rev Microbiol*. **1995**, 49, 711-745.
23. Zhang, Y. Persisters, persistent infections and the Yin-Yang model. *Emerg Microbes Infect*. **2014**, 3, e3.
24. Wood, T.K.; Knabel, S.J.; Kwan, B.W. Bacterial persister cell formation and dormancy. *Appl Environ Microbiol*. **2013**, 79, 7116-7121.
25. Lewis, K. Multidrug tolerance of biofilms and persister cells. *Curr Top Microbiol Immunol*. **2008**, 322, 107-131.
26. Zhang, Y.; Yew, W.W.; Barer, M.R. Targeting persisters for tuberculosis control. *Antimicrob Agents Chemother*. **2012**, 56, 2223-2230.
27. Zhang, Y. Persistent and dormant tubercle bacilli and latent tuberculosis. *Front Biosci*. **2004**, 9, 1136-1156.
28. Glover, W.A.; Yang, Y.; Zhang, Y. Insights into the molecular basis of L-form formation and survival in Escherichia coli. *PLoS One*. **2009**, 4, e7316.
29. Bjarnsholt T. The role of bacterial biofilms in chronic infections. *APMIS*. **2013**, 121 (Suppl. 136), 1–54.
30. Rolain, J.M.; Brouqui, P.; Koehler, J.E.; Maguina, C.; Dolan, M.J.; Raoult, D.

Recommendations for treatment of human infections caused by Bartonella species. *Antimicrob Agents Chemother*. **2004**, 48, 1921-1933.

31. Schulein, R.; Seubert, A.; Gille, C.; Lanz, C.; Hansmann, Y.; Piemont, Y.; Dehio, C. Invasion and persistent intracellular colonization of erythrocytes. A unique parasitic strategy of the emerging pathogen Bartonella. *J Exp Med*. **2001**, 193, 1077-1086.
32. Borchardt, J.K. The Beginnings of Drug Therapy: Ancient Mesopotamian Medicine. *Drug News Perspect*. **2002**, 15, 187-192.
33. Patwardhan, B.; Warude, D.; Pushpangadan, P.; Bhatt, N. Ayurveda and traditional Chinese medicine: a comparative overview. *Evid Based Complement Alternat Med*. **2005**, 2, 465-473.
34. Jaiswal, Y.; Liang, Z.; Zhao, Z. Botanical drugs in Ayurveda and Traditional Chinese Medicine. *J Ethnopharmacol*. **2016**, 194, 245-259.
35. Di Lorenzo, C.; Ceschi, A.; Kupferschmidt, H.; Lude, S.; De Souza Nascimento, E.; Dos Santos, A.; Colombo, F.; Frigerio, G.; Norby, K.; Plumb, J. et al. Adverse effects of plant food supplements and botanical preparations: a systematic review with critical evaluation of causality. *Br J Clin Pharmacol*. **2015**, 79, 578-592.
36. Garcia-Alvarez, A.; Mila-Villarroel, R.; Ribas-Barba, L.; Egan, B.; Badea, M.; Maggi, F.M.; Salmenhaara, M.; Restani, P.; Serra-Majem, L.; Plant, L.P.F.S.C.S.g. Usage of Plant Food Supplements (PFS) for weight control in six European countries: results from the PlantLIBRA PFS Consumer Survey 2011-2012. *BMC Complement Altern Med*. **2016**, 16, 254.
37. Lude, S.; Vecchio, S.; Sinno-Tellier, S.; Dopter, A.; Mustonen, H.; Vucinic, S.; Jonsson, B.; Muller, D.; Veras Gimenez Fruchtengarten, L.; Hruby, K. et al. Adverse Effects of Plant Food Supplements and Plants Consumed as Food: Results from the Poisons Centres-Based PlantLIBRA Study. *Phytother Res*. **2016**, 30, 988-996.
38. Popovic, V.B.; Tomic, M.A.; Stepanovic-Petrovic, R.M.; Micov, A.M.; Milenkovic, M.T.;

Petrovic, S.D.; Usjak, L.J.; Niketic, M.S. Laserpitium zernyi Hayek Flower and Herb Extracts: Phenolic Compounds, and Anti-edematous, Antioxidant, and Antimicrobial Activities. *Chem Biodivers*. **2017**, 14.

39. Denes, T.; Bartha, S.G.; Kerenyi, M.; Varga, E.; Balazs, V.L.; Csepregi, R.; Papp, N. Histological and antimicrobial study of Ononis arvensis L. *Acta Biol Hung*. **2017**, 68, 321-333.
40. Hickl, J.; Argyropoulou, A.; Sakavitsi, M.E.; Halabalaki, M.; Al-Ahmad, A.; Hellwig, E.; Aligiannis, N.; Skaltsounis, A.L.; Wittmer, A.; Vach, K. et al. Mediterranean herb extracts inhibit microbial growth of representative oral microorganisms and biofilm formation of Streptococcus mutans. *PLoS One*. **2018**, 13, e0207574.
41. Lee, M.S.; Choi, J.; Posadzki, P.; Ernst, E. Aromatherapy for health care: an overview of systematic reviews. *Maturitas*. **2012**, 71, 257-260.
42. Hyldgaard, M.; Mygind, T.; Meyer, R.L. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front. Microbiol*. **2012**, 3, 12.
43. Singh, G.; Kapoor, I.P.; Pandey, S.K.; Singh, U.K.; Singh, R.K. Studies on essential oils: part 10; antibacterial activity of volatile oils of some spices. *Phytother. Res*. **2002**, 16, 680-682.
44. Sakkas, H.; Gousia, P.; Economou, V.; Sakkas, V.; Petsios, S.; Papadopoulou, C. *In vitro* antimicrobial activity of five essential oils on multidrug resistant Gram-negative clinical isolates. *J. Intercult. Ethnopharmacol*. **2016**, 5, 212-218.
45. Langeveld, W.T.; Veldhuizen, E.J.; Burt, S.A. Synergy between essential oil components and antibiotics: a review. *Crit Rev Microbiol*. **2014**, 40, 76-94.
46. Feng, J.; Wang, T.; Zhang, S.; Shi, W.; Zhang, Y. An optimized SYBR Green I/PI assay for rapid viability assessment and antibiotic susceptibility testing for Borrelia burgdorferi. *PLoS One*. **2014**, 9, e111809.

47. Simpson, D.A.; Feeney, S.; Boyle, C.; Stitt, A.W. Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. *Mol Vis*. **2000**, 6, 178-183.
48. Morrison, T.B.; Weis, J.J.; Wittwer, C.T. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques*. **1998**, 24, 954-958, 960, 962.
49. Barbesti, S.; Citterio, S.; Labra, M.; Baroni, M.D.; Neri, M.G.; Sgorbati, S. Two and three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria. *Cytometry*. **2000**, 40, 214-218.
50. Nicoletti, I.; Migliorati, G.; Pagliacci, M.C.; Grignani, F.; Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods*. **1991**, 139, 271-279.
51. Feng, J.; Zhang, S.; Shi, W.; Zubcevik, N.; Miklossy, J.; Zhang, Y. Selective Essential Oils from Spice or Culinary Herbs Have High Activity against Stationary Phase and Biofilm *Borrelia burgdorferi*. *Front. Med (Lausanne)*. **2017**, 4, 169.
52. Feng, J.; Shi, W.; Miklossy, J.; Zhang, Y. Additional Essential Oils with High Activity against Stationary Phase Borrelia burgdorferi. *Bioarxiv* [preprint]. **2018**, 260091.
53. Feng, J.; Leone, J.; Schweig, S.; Zhang, Y. Evaluation of Natural and Botanical Medicines for Activity Against Growing and Non-growing Forms of B. burgdorferi. *Front Med (Lausanne)*. **2020**, 7, 6.
54. Feng, J.; Shi, W.; Zhang, S.; Sullivan, D.; Auwaerter, P.G.; Zhang, Y. A Drug Combination Screen Identifies Drugs Active against Amoxicillin-Induced Round Bodies of *In Vitro Borrelia burgdorferi* Persisters from an FDA Drug Library. *Front. Microbiol*. **2016**, 7, 743.
55. Li, T.; Feng, J.; Xiao, S.; Shi, W.; Sullivan, D.; Zhang, Y. Identification of FDA-Approved Drugs with Activity against Stationary Phase *Bartonella henselae*. *Antibiotics (Basel)*.

2019, 8.

56. Riess T.; Dietrich F.; Schmidt K.V.; Kaiser P.O.; Schwarz H.; Schäfer A.; Kempf V.A. Analysis of a novel insect cell culture medium-based growth medium for Bartonella species. *Appl Environ Microbiol*. **2008,** *74*, 5224-7.
57. Performance Standards for Antimicrobial Susceptibility Testing. Available online: https://clsi.org/standards/products/microbiology/documents/m100/ (accessed on 13 December 2018).
58. Goc, A.; Rath, M. The anti-borreliae efficacy of phytochemicals and micronutrients: an update. *Ther Adv Infect Dis*. **2016**, 3, 75-82.
59. Mills-Robertson, F.C.; Tay, S.C.; Duker-Eshun, G.; Walana, W.; Badu, K. In vitro antimicrobial activity of ethanolic fractions of Cryptolepis sanguinolenta. *Ann Clin Microbiol Antimicrob*. **2012**, 11, 16.
60. Marinas, I.C.; Oprea, E.; Chifiriuc, M.C.; Badea, I.A.; Buleandra, M.; Lazar, V. Chemical Composition and Antipathogenic Activity of Artemisia annua Essential Oil from Romania. *Chem Biodivers*. **2015**, 12, 1554-1564.
61. Ruiz-Ruiz, J.C.; Moguel-Ordonez, Y.B.; Segura-Campos, M.R. Biological activity of Stevia rebaudiana Bertoni and their relationship to health. *Crit Rev Food Sci Nutr*. **2017**, 57, 2680-2690.
62. Herrera, D.R.; Durand-Ramirez, J.E.; Falcao, A.; Silva, E.J.; Santos, E.B.; Gomes, B.P. Antimicrobial activity and substantivity of Uncaria tomentosa in infected root canal dentin. *Braz Oral Res*. **2016**, 30, e61.
63. Dorbecker, C.; Sander, A.; Oberle, K.; Schulin-Casonato, T. In vitro susceptibility of Bartonella species to 17 antimicrobial compounds: comparison of Etest and agar dilution. *J Antimicrob Chemother*. **2006**, 58, 784-788.
64. Ben Hsouna, A.; Ben Halima, N.; Smaoui, S.; Hamdi, N. *Citrus* lemon essential oil: chemical composition, antioxidant and antimicrobial activities with its preservative

effect against *Listeria monocytogenes* inoculated in minced beef meat. *Lipids. Health. Dis*. **2017**, 16, 146.

65. Aboelhadid, S.M.; Mahrous, L.N.; Hashem, S.A.; Abdel-Kafy, E.M.; Miller, R.J. *In vitro* and *in vivo* effect of *Citrus* limon essential oil against sarcoptic mange in rabbits. *Parasitol. Res*. **2016**, 115, 3013-3020.
66. Maurya, A.K.; Mohanty, S.; Pal, A.; Chanotiya, C.S.; Bawankule, D.U. The essential oil from *Citrus limetta* Risso peels alleviates skin inflammation: In-vitro and in-vivo study. *J. Ethnopharmacol*. **2018**, 212, 86-94.
67. Shen, C.Y.; Jiang, J.G.; Zhu, W.; Ou-Yang, Q. Anti-inflammatory Effect of Essential Oil from *Citrus aurantium L. var. amara* Engl. *J. Agric. Food. Chem*. **2017**, 65, 8586-8594.
68. Siti, H.N.; Kamisah, Y.; Nur Iliyani, M.I.; Mohamed, S.; Jaarin, K. Citrus leaf extract reduces blood pressure and vascular damage in repeatedly heated palm oil diet-Induced hypertensive rats. *Biomed. Pharmacother*. **2017**, 87, 451-460.
69. Bejaoui, A.; Chaabane, H.; Jemli, M.; Boulila, A.; Boussaid, M. Essential oil composition and antibacterial activity of *Origanum vulgare subsp. glandulosum* Desf. at different phenological stages. *J. Med. Food*. **2013**, 16, 1115-1120.
70. Shelef L.A.; Naglik O.A.; Bogen D.W. Sensitivity of some common food-borne bacteria to the spices sage, rosemary and allspice. *J. Food. Sci*. **1980**, 45, M1042-1044.
71. Ren, P.; Ren, X.; Cheng, L.; Xu, L. Frankincense, pine needle and geranium essential oils suppress tumor progression through the regulation of the AMPK/mTOR pathway in breast cancer. *Oncol. Rep*. **2018**, 39, 129-137.
72. Lysakowska, M.E.; Sienkiewicz, M.; Banaszek, K.; Sokolowski, J. The Sensitivity of Endodontic *Enterococcus* spp. Strains to Geranium Essential Oil. *Molecules*. **2015**, 20, 22881-22889.
73. Al-Yasiry, A.R.; Kiczorowska, B. Frankincense--therapeutic properties. *Postepy. Hig. Med. Dosw (Online)*. **2016**, 70, 380-391.

74. Ndoti-Nembe, A.; Vu, K.D.; Han, J.; Doucet, N.; Lacroix, M. Antimicrobial Effects of Nisin, Essential Oil, and gamma-Irradiation Treatments against High Load of *Salmonella* typhimurium on Mini-carrots. *J. Food. Sci*. **2015**, 80, M1544-1548.
75. Sue, C.; Gary, Y.; Craig, O.; Karen, N.; Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) by essential oils. *Flavour Fragr. J.* **2008**, 23, 444-449.
76. Ndoti-Nembe, A.; Vu, K.D.; Doucet, N.; Lacroix, M. Antimicrobial effects of essential oils, nisin, and irradiation treatments against *Listeria monocytogenes* on ready-to-eat carrots. *J. Food. Sci*. **2015**, 80, M795-799.
77. Broznic, D.; Ratkaj, I.; Malenica Staver, M.; Kraljevic Pavelic, S.; Zurga, P.; Bubalo, D.; Gobin, I. Evaluation of the Antioxidant Capacity, Antimicrobial and Antiproliferative Potential of Fir (*Abies alba Mill.*) Honeydew Honey Collected from Gorski kotar (Croatia). *Food. Technol. Biotechnol*. **2018**, 56, 533-545.
78. Gobin, I.; Crnkovic, G.; Magdalenic, M.; Begic, G.; Babic, A.; Lusic, D.; Vuckovic, D. Antibacterial potential of Croatian honey against antibiotic resistant pathogenic bacteria. *Med. Glas (Zenica)*. **2018**, 15, 139-144.
79. Whiley, H.; Gaskin, S.; Schroder, T.; Ross, K. Antifungal properties of essential oils for improvement of indoor air quality: a review. *Rev. Environ. Health*. **2018**, 33, 63-76.
80. Tan, L.T.; Lee, L.H.; Yin, W.F.; Chan, C.K.; Abdul Kadir, H.; Chan, K.G.; Goh, B.H. Traditional Uses, Phytochemistry, and Bioactivities of *Cananga odorata* (Ylang-Ylang). *Evid. Based. Complement. Alternat. Med*. **2015**, 2015, 896314.
81. Almeida Lde, F.; Paula, J.F.; Almeida, R.V.; Williams, D.W.; Hebling, J.; Cavalcanti, Y.W. Efficacy of citronella and cinnamon essential oils on *Candida albicans* biofilms. *Acta. Odontol. Scand*. **2016**, 74, 393-398.
82. Oliveira, J.B.; Teixeira, M.A.; Paiva, L.F.; Oliveira, R.F.; Mendonca, A.; Brito, M.J.A. *In Vitro* and *In Vivo* Antimicrobial Activity of *Cymbopogon citratus* (DC.) Stapf. Against *Staphylococcus* spp. Isolated from Newborn Babies in an Intensive Care Unit. *Microb.*

Drug. Resist. **2019**.

83. Mogana, R.; Teng-Jin, K.; Wiart, C. *In Vitro* Antimicrobial, Antioxidant Activities and Phytochemical Analysis of *Canarium patentinervium* Miq. from Malaysia. *Biotechnol. Res. Int*. **2011**, 2011, 768673.
84. David, A.; Wang, F.; Sun, X.; Li, H.; Lin, J.; Li, P.; Deng, G. Chemical Composition, Antioxidant, and Antimicrobial Activities of *Vetiveria zizanioides* (L.) Nash Essential Oil Extracted by Carbon Dioxide Expanded Ethanol. *Molecules*. **2019**, 24.
85. Powers, C.N.; Osier, J.L.; Mcfeeters, R.L.; Brazell, C.B.; Olsen, E.L.; Moriarity, D.M.; Satyal, P.; Setzer, W.N. Antifungal and Cytotoxic Activities of Sixty Commercially-Available Essential Oils. *Molecules*. **2018**, 23.
86. Bi, D.; Zhao, Y.; Jiang, R.; Wang, Y.; Tian, Y.; Chen, X.; Bai, S.; She, G. Phytochemistry, Bioactivity and Potential Impact on Health of Juglans: the Original Plant of Walnut. *Nat Prod Commun*. **2016**, 11, 869-880.
87. Catanzaro, E.; Greco, G.; Potenza, L.; Calcabrini, C.; Fimognari, C. Natural Products to Fight Cancer: A Focus on Juglans regia. *Toxins (Basel)*. **2018**, 10.
88. Wianowska, D.; Garbaczewska, S.; Cieniecka-Roslonkiewicz, A.; Dawidowicz, A.L.; Jankowska, A. Comparison of antifungal activity of extracts from different Juglans regia cultivars and juglone. *Microb Pathog*. **2016**, 100, 263-267.
89. Karvonen, K.; Gilbert, L. Effective killing of Borrelia burgdorferi in vitro with novel herbal compounds. *General Medicine Open*. **2018**, 2.
90. Panth, N.; Paudel, K.R.; Karki, R. Phytochemical profile and biological activity of Juglans regia. *Journal of Integrative Medicine*. **2016**, 14, 359-373.
91. Hayes, D.; Angove, M.J.; Tucci, J.; Dennis, C. Walnuts (Juglans regia) Chemical Composition and Research in Human Health. *Crit Rev Food Sci Nutr*. **2016**, 56, 1231-1241.
92. Ahmad, T.; Suzuki, Y.J. Juglone in Oxidative Stress and Cell Signaling. *Antioxidants*

(Basel). **2019**, 8.

93. Zmantar, T.; Miladi, H.; Kouidhi, B.; Chaabouni, Y.; Ben Slama, R.; Bakhrouf, A.; Mahdouani, K.; Chaieb, K. Use of juglone as antibacterial and potential efflux pump inhibitors in Staphylococcus aureus isolated from the oral cavity. *Microb Pathog*. **2016**, 101, 44-49.
94. Ho, K.V.; Lei, Z.; Sumner, L.W.; Coggeshall, M.V.; Hsieh, H.Y.; Stewart, G.C.; Lin, C.H. Identifying Antibacterial Compounds in Black Walnuts (Juglans nigra) Using a Metabolomics Approach. *Metabolites*. **2018**, 8.
95. Ozcelik, B.; Kartal, M.; Orhan, I. Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. *Pharm Biol*. **2011**, 49, 396-402.
96. Paudel, P.; Satyal, P.; Dosoky, N.S.; Maharjan, S.; Setzer, W.N. Juglans regia and J. nigra, two trees important in traditional medicine: A comparison of leaf essential oil compositions and biological activities. *Nat Prod Commun*. **2013**, 8, 1481-1486.
97. Forkuo, A.D.; Ansah, C.; Mensah, K.B.; Annan, K.; Gyan, B.; Theron, A.; Mancama, D.; Wright, C.W. In vitro anti-malarial interaction and gametocytocidal activity of cryptolepine. *Malar J*. **2017**, 16, 496.
98. Tona, L.; Kambu, K.; Ngimbi, N.; Cimanga, K.; Vlietinck, A.J. Antiamoebic and phytochemical screening of some Congolese medicinal plants. *J Ethnopharmacol*. **1998**, 61, 57-65.
99. Cimanga, K.; De Bruyne, T.; Pieters, L.; Totte, J.; Tona, L.; Kambu, K.; Berghe, D.V.; Vlietinck, A.J. Antibacterial and antifungal activities of neocryptolepine, biscryptolepine and cryptoquindoline, alkaloids isolated from Cryptolepis sanguinolenta. *Phytomedicine*. **1998**, 5, 209-214.
100. Hanprasertpong, N.; Teekachunhatean, S.; Chaiwongsa, R.; Ongchai, S.; Kunanusorn, P.; Sangdee, C.; Panthong, A.; Bunteang, S.; Nathasaen, N.; Reutrakul, V. Analgesic, anti-inflammatory, and chondroprotective activities of Cryptolepis

buchanani extract: in vitro and in vivo studies. *Biomed Res Int*. **2014**, 2014, 978582.

101. Ansah, C.; Mensah, K.B. A review of the anticancer potential of the antimalarial herbal cryptolepis sanguinolenta and its major alkaloid cryptolepine. *Ghana Med J*. **2013**, 47, 137-147.

102. Osafo, N.; Mensah, K.B.; Yeboah, O.K. Phytochemical and Pharmacological Review of Cryptolepis sanguinolenta (Lindl.) Schlechter. *Adv Pharmacol Sci*. **2017**, 2017, 3026370.

103. Sawer, I.K.; Berry, M.I.; Ford, J.L. The killing effect of cryptolepine on Staphylococcus aureus. *Lett Appl Microbiol*. **2005**, 40, 24-29.

104. Lisgarten, J.N.; Coll, M.; Portugal, J.; Wright, C.W.; Aymami, J. The antimalarial and cytotoxic drug cryptolepine intercalates into DNA at cytosine-cytosine sites. *Nat Struct Biol*. **2002**, 9, 57-60.

105. Pal, H.C.; Katiyar, S.K. Cryptolepine, a Plant Alkaloid, Inhibits the Growth of Non-Melanoma Skin Cancer Cells through Inhibition of Topoisomerase and Induction of DNA Damage. *Molecules*. **2016**, 21.

106. Kim, J.R.; Oh, D.R.; Cha, M.H.; Pyo, B.S.; Rhee, J.H.; Choy, H.E.; Oh, W.K.; Kim, Y.R. Protective effect of polygoni cuspidati radix and emodin on Vibrio vulnificus cytotoxicity and infection. *J Microbiol*. **2008**, 46, 737-743.

107. Song, J.H.; Kim, S.K.; Chang, K.W.; Han, S.K.; Yi, H.K.; Jeon, J.G. In vitro inhibitory effects of Polygonum cuspidatum on bacterial viability and virulence factors of Streptococcus mutans and Streptococcus sobrinus. *Arch Oral Biol*. **2006**, 51, 1131-1140.

108. Pandit, S.; Kim, H.J.; Park, S.H.; Jeon, J.G. Enhancement of fluoride activity against Streptococcus mutans biofilms by a substance separated from Polygonum cuspidatum. *Biofouling*. **2012**, 28, 279-287.

109. Wu, X.; Li, Q.; Feng, Y.; Ji, Q. Antitumor Research of the Active Ingredients from

Traditional Chinese Medical Plant Polygonum Cuspidatum. *Evid Based Complement Alternat Med*. **2018**, 2018, 2313021.

110. Zhang, H.; Li, C.; Kwok, S.T.; Zhang, Q.W.; Chan, S.W. A Review of the Pharmacological Effects of the Dried Root of Polygonum cuspidatum (Hu Zhang) and Its Constituents. *Evid Based Complement Alternat Med*. **2013**, 2013, 208349.

111. Breuss, J.M.; Atanasov, A.G.; Uhrin, P. Resveratrol and Its Effects on the Vascular System. *Int J Mol Sci*. **2019**, 20.

112. Abdelgawad, I.Y.; Grant, M.K.O.; Zordoky, B.N. Leveraging the Cardio-Protective and Anticancer Properties of Resveratrol in Cardio-Oncology. *Nutrients*. **2019**, 11.

113. Vestergaard, M.; Ingmer, H. Antibacterial and antifungal properties of resveratrol. *Int J Antimicrob Agents*. **2019**, 53, 716-723.

114. Feng, J.; Shi, W.; Zhang, S.; Zhang, Y. Identification of new compounds with high activity against stationary phase Borrelia burgdorferi from the NCI compound collection. *Emerg Microbes Infect*. **2015**, 4, e31.

115. Pan, B.; Shi, X.; Ding, T.; Liu, L. Unraveling the action mechanism of polygonum cuspidatum by a network pharmacology approach. *Am J Transl Res*. **2019**, 11, 6790-6811.

116. La Porte, C.; Voduc, N.; Zhang, G.; Seguin, I.; Tardiff, D.; Singhal, N.; Cameron, D.W. Steady-State pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (ethanol) in healthy human subjects. *Clin Pharmacokinet*. **2010**, 49, 449-454.

117. Maaland, M.G.; Papich, M.G.; Turnidge, J.; Guardabassi, L. Pharmacodynamics of doxycycline and tetracycline against Staphylococcus pseudintermedius: proposal of canine-specific breakpoints for doxycycline. *J Clin Microbiol*. **2013**, 51, 3547-3554.

118. Lode, H.; Borner, K.; Koeppe, P.; Schaberg, T. Azithromycin--review of key chemical, pharmacokinetic and microbiological features. *J Antimicrob Chemother*.

1996, 37 Suppl C, 1-8.

119. Zhang, Y.; Shi, W.; Zhang, W.; Mitchison, D. Mechanisms of pyrazinamide action and resistance. *Microbiol Spectr*. **2013**, 2(4), 1-12.

120. Feng, J.; Wang, T.; Shi, W.; Zhang, S.; Sullivan, D.; Auwaerter P.G.; Zhang, Y. Identification of novel activity against Borrelia burgdorferi persisters using an FDA approved drug library. *Emerg Microbes Infect.* **2014**, 3(7), e49.

121. Zouhir, A.; Jridi, T.; Nefzi, A.; Ben Hamida, J.; Sebei, K. Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) by antimicrobial peptides (AMPs) and plant essential oils. *Pharm. Biol*. **2016**, 54, 3136-3150.

122. Gadisa, E.; Weldearegay, G.; Desta, K.; Tsegaye, G.; Hailu, S.; Jote, K.; Takele, A. Combined antibacterial effect of essential oils from three most commonly used Ethiopian traditional medicinal plants on multidrug resistant bacteria. *BMC. Complement. Altern. Med*. **2019**, 19, 24.

123. Orchard, A.; Van Vuuren, S. F.; Viljoen, A.M.; Kamatou, G. The *in vitro* antimicrobial evaluation of commercial essential oils and their combinations against acne. *Inter. J. Cosmet. Sci*, **2018**, 40, 226–243.

124. Lanzerstorfer, A.; Hackl, M.; Schlomer, M.; Rest, B.; Deutsch-Grasl, E.; Lanzerstorfer, C. The influence of air-dispersed essential oils from lemon (Citrus limon) and silver fir (Abies alba) on airborne bacteria and fungi in hospital rooms. *J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng*. **2019**, 54, 256-260.

125. Costerton, J.W.; Stewart, P.S.; Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. *Science*. **1999**, 284, 1318-1322.

126. Wolfmeier, H.; Pletzer, D.; Mansour, S.C.; Hancock, R.E.W. New Perspectives in Biofilm Eradication. *ACS Infect Dis*. **2018**, 4, 93-106.

127. Breitschwerdt, E.B.; Kordick, D.L. *Bartonella* infection in animals: carriership, reservoir potential, pathogenicity, and zoonotic potential for human infection. *Clin.*

Microbiol. Rev. **2000**, 13, 428-438.

SUPPLEMENTARY

Table S1. Herbal product sources, validation, and extract details

Herbal Product	Plant or Ingredients of Herbal Products	Source	Validation/ID	Extract Details
Hu tao ren	*Juglans nigra* fruit (husk/hull)	Pacific Botanicals (Wild harvested)	Organoleptic, KW Botanicals	45% ETOH extract of husk/hulls by KW Botanicals
Black walnut	*Juglans nigra*	Heron Botanicals (Wild harvested, New York)	Organoleptic, Heron Botanicals	30, 60, 90% ETOH by Heron Botanicals
Coptis	*Rhizoma coptidis*	Hawaii Pharm LLC (Certified organic coptis)		Root extract by Hawaii Pharm LLC (Honolulu, HI)
Japanese knotweed	*Polygonum cuspidatum*	Heron Botanicals (Organic cultivation, China)	Organoleptic, Heron Botanicals	30, 60, 90% ETOH by Heron Botanicals
Tian ju ye	*Stevia rebaudiana*	Sonoma County Herb Exchange (Cultivated)	Organoleptic, KW Botanicals	25% ETOH extract by KW Botanicals
Samento	*Uncaria tomentosa* bark	NutraMedix, LLC (Jupiter, Florida)		Samento bark 20-24% ETOH extract
Grapefruit seed extract	*Citrus paradisi*	Cintamani, Poland (Citrosept ™)	Cintamani, Poland	Organic grapefruit seed extract
Chuan xin lian	*Andrographis paniculata*	Heron Botanicals (Organic cultivation, China)	Organoleptic, Heron Botanicals	30, 60, 90% ETOH by Heron Botanicals
Sweet wormwood	*Artemisia annua*	Heron Botanicals (Organic cultivation)	American Herbal Pharmacopoeia (Scotts Valley, CA), Organoleptic, Heron Botanicals Confirmed 0.11% Artemisinin content, The Institute for Food Safety and Defense	30, 60, 90% ETOH by Heron Botanicals

Banderol	*Otoba* spp. bark	NutraMedix, LLC (Jupiter, Florida)		Banderol bark 20-24% ETOH extract
Hu zhang	*Polygonum cuspidatum*	Hawaii Pharm LLC (Wildcrafted)		Root extract by Hawaii Pharm LLC (Honolulu, HI)
Cryptolepis	*Cryptolepis sanguinolenta*	Heron Botanicals (Wild harvested, Ghana)	HPTLC, The Institute for Food Safety and Defense Organoleptic, Heron Botanicals	30, 60, 90% ETOH by Heron Botanicals
Huang qin	*Scutellaria baicalensis*	Heron Botanicals (Organic cultivation, China)	Organoleptic, Heron Botanicals	30, 60, 90% ETOH by Heron Botanicals
Ban zhi lian	*Scutellaria barbata*	Hawaii Pharm LLC (Wild harvested, China)		Dried herb powder extracted by Hawaii Pharm LLC (Honolulu, HI)
Cumanda	*Campsiandra angustifolia* bark	NutraMedix, LLC (Jupiter, Florida)		Cumanda bark 20-24% ETOH extract
Gou Teng	*Uncaria rhynchophylla*	Hawaii Pharm LLC		Stalk extract by Hawaii Pharm LLC (Honolulu, HI)
Gao liang jiang	*Dipsacus fullonum*	Friend's of the Trees (wild harvested, Washington State)	DNA Species Identification, NSF International	40% ETOH by KW Botanicals (Inadvertently comingled with D. asper sample prior to testing)
Uncaria	*Uncaria tomentosa*	Mountain Rose Herbs (Wild harvested)	DNA Species Identification, Christopher Hobbs, Ph.D.	50% ETOH by KW Botanicals

Table S2. Chemical compositions of top hit essential oils against *B. henselae*

Essential oils	Plant of essential oils	Plant part	Main chemical composition	References
Cinnamon bark	*Cinnamomum zeylanicum*	bark	cinnamaldehyde, camphene, 1,8-cineole, α-terpineol, bornyl acetate, etc.	[1]
Oregano	*Origanum vulgare hirtum*	herbs	thymol, γ-terpinene, carvacrol, p-cymene, myrcene, etc.	[2]
Elemi	*Canarium luzonicum*	resin	elemol, elemecin, sabinene, α-phellandrene, p-cymene, limonene, α-terpineol, etc.	[3]
Mountain savory (winter)	*Satureja montana*	herbs	carvacrol, borneol, carvacrylacetate, α-terpineol, thymol, etc.	[4]
Cedarwood	*Cedrus deodora*	wood	wikstromol, matairesinol, benzylbutyrolactol, BDFD	[5]
Ylang ylang	*Cananga odorata*	flowers	linalool, β-caryophyllene, α-humulene, γ-muurolene, germacrene D, (3E,6E)-α-farnesene, δ-cadinene, benzyl benzoate	[6]
Citronella	*Cymbopogon winterianus*	leaves	citronellal, isomenthone, citronellol, geraniol, limonene, etc.	[7]
Clove bud 1	*Eugenia caryophyllata*	bud	eugenol, eugenyl acetate, β-caryophyllene, carvacrol, cinnamaldehyde, thymol, etc.	[8]
Clove bud 2	*Syzygium aromaticum L*	bud	eugenol, β-caryophyllene, eugenyl acetate, etc.	[9]
Geranium bourbon	*Pelargonium graveolens*	leaves and flowers	linalool, citronellol, iso-menthone, geraniol, citronellyl formate, geraniol formate, etc.	[10]
Allspice	*Pimenta officinalis*	berries	eugenol, methyleugenol, β-caryophyllene, α-humulene, etc.	[11]
Vetiver	*Vetiveria zizanoides*	root	cycloisolongifolene, khusimene, β-cadinene, β-guaiene, β-vetivenene, etc.	[12]
Cinnamon leaf	*Cinnamomum zeylanicum*	leaf	eugenol, benzyl benzoate, linalool, cinnamaldehyde, etc.	[13]
Geranium	*Pelargonium asperum*	herbs	citronellol, citronellyl ester, geraniol, buthyl anthranilate, etc.	[14]
Bergamot	*Citrus bergamia*	fruit peel	limonene, β-pinene, γ-terpinene, linalool, linalyl acetate, etc.	[15]
Cajeput	*Melaleuca cajeputi*	leaves and buds	ethanone, 4H-1-benzopyran-4-one, 1,4-naphthalenedione, naphthalene, etc.	[16]
Marjoram (sweet)	*Origanum majorana*	leaves and flowers	α-terpineol, (-)-Terpinen-4-ol, 5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol, β-cymene, β-phellandrene, etc.	[17]

Fir needle	*Abies siberica*	needles	α-pinene, camphene, β-pinene, Δ3-carene, limonene, bornyl acetate, etc.	[18]
Grapefruit	*Citrus paradisi*	peel	limonene, β-myrcene, α-pinene, etc.	[19]
Spearmint	*Mentha spicata*	flowering herbs	carvone, limonene, 1,8-cineole, β-caryophyllene, germacrene D, etc.	[20]
Tangerine	*Citrus reticulata*	fruit peel	citronellal, thymol, geranyl acetate, β-elemen, germacrene B, etc.	[21]
Tea tree	*Melaleuca alternifolia*	leaves	α-pinene, α-terpinene, 1,8-cineole, γ-terpinene, terpinolene, terpinen-4-ol, α-terpineol, etc.	[22]
Lemon	*Citrus limonum*	peel	β-pinene, limonene, linalool, α-terpineol, linalyl acetate, nerolidol, farnesol, etc.	[23]
Ho wood	*Cinnamomum camphora*	twigs and bark	D-camphor, 1,8-cineole, α-terpineol, while D-camphor, linalool, 1,8-cineole, etc.	[24]
Frankincense	*Boswellia serrata*	resin	α-pinene, α-thujene, methylchavicol, sabinene, methyleugenol, myrcene, limonene, p-cymene, etc.	[25]

References of Supplementary

1. Falleh, H.; Ben Jemaa, M.; Djebali, K.; Abid, S.; Saada, M.; Ksouri, R. Application of the mixture design for optimum antimicrobial activity: Combined treatment of Syzygium aromaticum, Cinnamomum zeylanicum, Myrtus communis, and Lavandula stoechas essential oils against Escherichia coli. *J. Food. Process. Preserv*. **2019**, 00: e14257.
2. Vinciguerra, V.; Rojas, F.; Tedesco, V.; Giusiano, G.; Angiolella, L. Chemical characterization and antifungal activity of Origanum vulgare, Thymus vulgaris essential oils and carvacrol against Malassezia furfur. *Nat. Prod. Res*. **2019**, 33, 3273-3277.
3. Nikolic, M.; Smiljkovic, M.; Markovic, T.; Cirica, A.; Glamoclija, J.; Markovic, D.; Sokovic, M. Sensitivity of clinical isolates of Candida to essential oils from Burseraceae family. *EXCLI J*. **2016**, 15, 280-289.
4. Santos, J.D.C.; Coelho, E.; Silva, R.; Passos, C.P.; Teixeira, P.; Henriques, I.; Coimbra, M.A. Chemical composition and antimicrobial activity of Satureja montana byproducts essential oils. *Industrial Crops and Products*. **2019**, 137, 541-548.
5. Dhayabaran, D.; Florance, E.J.; Nandakumar, K.; Shanmugarathinam, A.; Puratchikody, A. Anticonvulsant activity of fraction isolated from ethanolic extract of heartwood of Cedrus

deodara. *J. Nat. Med*. **2014**, 68, 310-315.

6. Compositions of essential oils Available online: https://www.theplantguru.com/amfile/file/download/file_id/28/product_id/734/ (accessed on 8 November 2019)
7. Compositions of essential oils Available online: https://www.theplantguru.com/amfile/file/download/file_id/9/product_id/714/ (accessed on 8 November 2019)
8. Chaieb, K.; Hajlaoui, H.; Zmantar, T.; Kahla-Nakbi, A.B.; Rouabhia, M.; Mahdouani, K.; Bakhrouf, A. The chemical composition and biological activity of clove essential oil, Eugenia caryophyllata (Syzigium aromaticum L. Myrtaceae): a short review. *Phytother. Res*. **2007**, 21, 501-506.
9. Compositions of essential oils Available online: https://www.theplantguru.com/amfile/file/download/file_id/11/product_id/726/ (accessed on 8 November 2019)
10. Essid, R.; Hammami, M.; Gharbi, D.; Karkouch, I.; Hamouda, T.B.; Elkahoui, S.; Limam, F.; Tabbene, O. Antifungal mechanism of the combination of Cinnamomum verum and Pelargonium graveolens essential oils with fluconazole against pathogenic Candida strains. *Appl. Microbiol. Biotechnol*. **2017**, 101, 6993-7006.
11. Compositions of essential oils Available online: https://www.theplantguru.com/amfile/file/download/file_id/1/product_id/622/ (accessed on 8 November 2019)
12. David, A.; Wang, F.; Sun, X.; Li, H.; Lin, J.; Li, P.; Deng, G. Chemical Composition, Antioxidant, and Antimicrobial Activities of Vetiveria zizanioides (L.) Nash Essential Oil Extracted by Carbon Dioxide Expanded Ethanol. *Molecules*. **2019**, 24.
13. Dongmo, P.M.J.; Tatsadjieu, L.N.; Tchoumbougnang, F.; Sameza, M.L.; Dongmo, B.N.; Zollo, P.H.A.; Menut, C. Chemical Composition, Antiradical and Antifungal Activities of Essential Oil of the Leaves of Cinnamomum Zeylanicum Blume from Cameroon. *Natural Product Communications*. **2007**, 2.
14. Ouedrhiri, W.; Balouiri, M.; Bouhdid, S.; Harki, E.H.; Moja, S.; Greche, H. Antioxidant and antibacterial activities of Pelargonium asperum and Ormenis mixta essential oils and their

synergistic antibacterial effect. *Environ. Sci. Pollut. Res. Int*. **2018**, 25, 29860-29867.

15. Al-Abd, N.M.; Mohamed Nor, Z.; Mansor, M.; Azhar, F.; Hasan, M.S.; Kassim, M. Antioxidant, antibacterial activity, and phytochemical characterization of Melaleuca cajuputi extract. *BMC. Complement. Altern. Med*. **2015**, 15, 385.
16. Compositions of essential oils Available online: https://www.theplantguru.com/amfile/file/download/file_id/5/product_id/646/ (accessed on 8 November 2019)
17. Makrane, H.; Aziz, M.; Berrabah, M.; Mekhfi, H.; Ziyyat, A.; Bnouham, M.; Legssyer, A.; Elombo, F.K.; Gressier, B.; Eto, B. Myorelaxant Activity of essential oil from Origanum majorana L. on rat and rabbit. *J. Ethnopharmacol*. **2019**, 228, 40-49.
18. Ou, M.C.; Liu, Y.H.; Sun, Y.W.; Chan, C.F. The Composition, Antioxidant and Antibacterial Activities of Cold-Pressed and Distilled Essential Oils of Citrus paradisi and Citrus grandis (L.) Osbeck. *Evid. Based. Complement. Alternat. Med*. **2015**, 2015, 804091.
19. Compositions of essential oils Available online: https://www.theplantguru.com/amfile/file/download/file_id/15/product_id/810/ (accessed on 8 November 2019)
20. Bardaweel, S.K.; Bakchiche, B.; Ha, A.L.; Rezzoug, M.; Gherib, A.; Flamini, G. Chemical composition, antioxidant, antimicrobial and Antiproliferative activities of essential oil of Mentha spicata L. (Lamiaceae) from Algerian Saharan atlas. *BMC. Complement. Altern. Med*. **2018**, 18, 201.
21. Duan, L.; Guo, L.; Dou, L.L.; Zhou, C.L.; Xu, F.G.; Zheng, G.D.; Li, P.; Liu, E.H. Discrimination of Citrus reticulata Blanco and Citrus reticulata 'Chachi' by gas chromatograph-mass spectrometry based metabolomics approach. *Food. Chem*. **2016**, 212, 123-127.
22. Ben Hsouna, A.; Ben Halima, N.; Smaoui, S.; Hamdi, N. Citrus lemon essential oil: chemical composition, antioxidant and antimicrobial activities with its preservative effect against Listeria monocytogenes inoculated in minced beef meat. *Lipids. Health. Dis*. **2017**, 16, 146.
23. Compositions of essential oils Available online: https://www.theplantguru.com/amfile/file/download/file_id/26/product_id/998/ (accessed on 8 November 2019)
24. Guo, S.; Geng, Z.; Zhang, W.; Liang, J.; Wang, C.; Deng, Z.; Du, S. The Chemical Composition

of Essential Oils from Cinnamomum camphora and Their Insecticidal Activity against the Stored Product Pests. *Int. J. Mol. Sci*. **2016**, 17.

25. Al-Yasiry, A.R.; Kiczorowska, B. Frankincense--therapeutic properties. *Postepy. Hig. Med. Dosw (Online)*. **2016**, 70, 380-391.

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